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PROGRESS REPORT

of grant AFOSR-89-0231

January 1992

by:

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Project Director

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92-05574



92 3 03 047

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE		3. REPORT TYPE AND DATES COVERED FINAL 01 Jan 89 TO 31 Dec 91	
4. TITLE AND SUBTITLE MOLECULAR TOXICOLOGY OF CHROMATIN				5. FUNDING NUMBERS AFOSR-89-0231 PE - 61102F PR - 2312 TA - A5	
6. AUTHOR(S) Dr Ernest Kun					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Romberg Tiburon Centers Center for Environmental Studies San Francisco State University P.O. Box 855, 3150 Paradise Drive Tiburon, CA 94920				8. PERFORMING ORGANIZATION REPORT NUMBER AFOSR-TR-92-0092	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Dr Jimmy Cornette AFOSR/NL Building 410 Bolling AFB DC 20332-6448				10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION/AVAILABILITY STATEMENT unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The binding of the ADP-ribosyl transferase protein and its polypeptide components, obtained by digestion with plasmin, to histone-Sepharose 4B matrices was determined by a centrifugation technique. Both the intact enzyme protein and the 29 kDa terminal polypeptide bound to histone affinity matrices in a strictly DNA-dependent manner. Whereas the nature of covalently matrix-bound histones had no apparent specificity towards the enzyme protein or its polypeptide components, among the polypeptides only the 29 kDa terminal basic polypeptide associated with the histone affinity matrices in a DNA-dependent manner. The binding properties of spermine-, polylysine- and polyarginine-Sepharose 4B affinity matrices were also determined. The spermine matrix exhibited similarities to the histone affinity matrix, except binding was considerably weaker, whereas affinity matrices of synthetic polyamino acids showed individual variations but did not replace histones as affinity ligands. The catalytic significance of histone-enzyme associations was tested by determining the effects of the polypeptide components on the enzymatic ADP-ribosyl transferase reaction in the presence of a synthetic octadeoxyribonucleotide duplex as coenzyme. The 29 kDa polypeptide exhibited competitive inhibition towards histones and the synthetic DNA analog,					
14. SUBJECT TERMS				15. NUMBER OF PAGES	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT (U)		18. SECURITY CLASSIFICATION OF THIS PAGE (U)		19. SECURITY CLASSIFICATION OF ABSTRACT (U)	
				20. LIMITATION OF ABSTRACT (U)	

ONLY. The binding constant of histones and inhibitory constant of the 29 kDa polypeptide towards the activation by histones were in the same order of magnitude, whereas proteolytic cleavage of the DNA binding domain of the enzyme into 29 and 36 kDa polypeptides decreased their affinity towards DNA 60-fold. The polycations had a characteristic catalytic activating effect on the ADP-ribosyl transferase enzymatic rates. Activation by histones was maximal with the synthetic DNA analog (octamer C) as coenzyme, whereas the largest in vitro activation by spermine occurred when the naturally occurring enzyme-bound DNA served as coenzyme. In no instance was histone ADP-ribosylated during the activation of ADP-ribosyl transferase by histones when the reaction was carried out in solution, but extensive histone ADP-ribosylation took place in reconstructed nucleosomes. The decay of ADP-ribosyl transferase activity which occurred upon dissociation by dilution to monomers was prevented by histones. It is suggested that the binding of physiologically occurring polycations to the 29 kDa polypeptide of the enzyme protein represents a biologically meaningful regulatory system for ADP-ribosyl transferase in vivo which can influence DNA conformation.

PUBLICATIONS IN 1991

1. Kirsten, E., Bauer, P.I. and Kun, E. (1991) "Suppression of Dexamethasone Stimulated DNA Synthesis in an Oncogene Construct Containing Rat Cell Line by a DNA-site Oriented Ligand of Poly (ADP-Ribose) Polymerase" *Exper. Cell Res.* 193, 1-4.
2. Bauer, P.I., Kline, K. and Kun, E. (1991) "Apparent Role of Adenosine Diphosphoribosyl Transferase in the Development of Mytilus Edulis and the Inhibition of Differentiation by Ligands of the Enzyme Protein" *Proc. Soc. Exper. Biol. Med.* 196, 396-400.
3. Kirsten, E., Bauer, P.I. and Kun, E. (1991) "Cellular Regulation of ADP-Ribosylation of Proteins. IV. Conversion of Poly(ADP-Ribose) Polymerase Activity to NAD Glycohydrolase During Retinoic Acid Induced Differentiation of HL60 Cells" *Exper. Cell Res.* 194, 1-8.
4. Buki, K.G., Bauer, P.I., Mendeleyev, J., Hakam, A. and Kun, E. (1991) "Destabilization of Zn(II) Coordination in ADP-Ribose Transferase (Polymerizing) by 6-Nitroso-1,2-benzopyrone Coincidental with Inactivation of the Polymerase but not the DNA Binding Function" *FEBS Lett.* 280, 181-185.
5. Buki, K.G., Bauer, P.I. and Kun, E. (1991) "Inhibitory Binding of Adenosine Diphosphoribosyl Transferase to the DNA Primer Site of Reverse Transcriptase Templates" *Biochem. Biophys. Res. Commun.* 180, 496-503.
6. Cole, G.A., Bauer, G., Kirsten, E., Mendeleyev, J., Bauer, P.I., Buki, K.G., Hakam, A and Kun, E. (1991) "Inhibition of HIV-1 IIIb Replication in AA-2 and MT-2 Cells in Culture by Two Ligands of Poly(ADP-Ribose) Polymerase: 6-Amino-1,2-benzopyrone and 5-Iodo-6-amino-1,2-benzopyrone" *Biochem. Biophys. Res. Commun.* 180, 504-514.

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- III. Inhibitory Binding of Adenosine Diphosphoribosyl Transferase to the DNA Primer Site of Reverse Transcriptase Templates.

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The Binding of Histones, Spermine, Polylysine and Polyarginine to ADP-Ribosyl Transferase, Analysis of the Histone Binding Polypeptide Site of the Enzyme and the Catalytic Effects of Polycations on the Enzymatic Reaction.

Abstract

The binding of the ADP-ribosyl transferase protein and its polypeptide components, obtained by digestion with plasmin, to histone-Sepharose 4B matrices was determined by a centrifugation technique. Both the intact enzyme protein and the 29 kDa terminal polypeptide bound to histone affinity matrices in a strictly DNA-dependent manner. Whereas the nature of covalently matrix-bound histones had no apparent specificity towards the enzyme protein or its polypeptide components, among the polypeptides only the 29 kDa terminal basic polypeptide associated with the histone affinity matrices in a DNA-dependent manner. The binding properties of spermine-, polylysine- and polyarginine-Sepharose 4B affinity matrices were also determined. The spermine matrix exhibited similarities to the histone affinity matrix, except binding was considerably weaker, whereas affinity matrices of synthetic polyamino acids showed individual variations but did not replace histones as affinity ligands. The catalytic significance of histone-enzyme associations was tested by determining the effects of the polypeptide components on the enzymatic ADP-ribosyl transferase reaction in the presence of a synthetic octadeoxyribonucleotide duplex as coenzyme. The 29 kDa polypeptide exhibited competitive inhibition towards histones and the synthetic DNA analog, whereas the 36 kDa polypeptide competitively inhibited the activation by the DNA analog only. The binding constant of histones and inhibitory constant of the 29 kDa polypeptide towards the activation by histones were in the same order of magnitude, whereas proleptic cleavage of the DNA

binding domain of the enzyme into 29 and 36 kDa polypeptides decreased their affinity towards DNA 60-fold. The polycations had a characteristic catalytic activating effect on the ADP-ribosyl transferase enzymatic rates. Activation by histones was maximal with the synthetic DNA analog (octamer C) as coenzyme, whereas the largest in vitro activation by spermine occurred when the naturally occurring enzyme-bound DNA served as coenzyme. In no instance was histone ADP-ribosylated during the activation of ADP-ribosyl transferase by histones when the reaction was carried out in solution, but extensive histone ADP-ribosylation took place in reconstructed nucleosomes. The decay of ADP-ribosyl transferase activity which occurred upon dissociation by dilution to monomers was prevented by histones. It is suggested that the binding of physiologically occurring polycations to the 29 kDa polypeptide of the enzyme protein represents a biologically meaningful regulatory system for ADP-ribosyl transferase in vivo which can influence DNA conformation.

Introduction

The DNA associated non-histone nuclear protein, poly (ADP-ribose) polymerase or transferase (E.C.2.4.2.30, abbreviated as ADPRT) was discovered on the basis its enzymatic activity which consists of the polymerization of the ADPR moiety of NAD^+ to a branched (Kanai et al. 1982) and helical (Minaga and Kun 1983a,b) homopolymer, that is covalently bound to the ADPRT molecule and to certain other nuclear proteins, for example to histones (see reviews Ueda and Hayaishi, 1985; Althaus and Richter 1987). Part of the catalytic reaction is shown in the equation



where NA is nicotinamide. In this reaction ADPRT plays the unusual role of being the catalyst as well as a reactant and (dsDNA) serves as a "coenzyme", which has certain structural requirements for coenzymic function, such as single- or double-stranded cuts (Benjamin and Gill 1980a,b) or the existence of replication origins (de Murcia and Jangstra-Bilen 1983) or both. In agreement with the concept of a coenzyme, DNA has been thought not to undergo any changes in the course of the reaction shown in the equation. However recent evidence indicates that this may not be so, since structural alterations ensue in DNA following the binding of ADPRT, and these topological changes occur even without the initiation of the polymerization of ADPR, since they take place with circular DNAs also which contain no nicks (Sastry and Kun 1988, 1989). The equation falls short in explaining

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poly(ADP-ribosylation) of proteins other than ADPRT and we proposed a trans-ADP-ribosylation mechanism that may involve a highly base-unstable ADPRT-ADPR intermediate (Bauer et al. 1986), but details of this process are as yet missing. It has become increasingly evident that in cells not exposed to toxic DNA damage the polymerase activity of ADPRT represents only 1% of the molecular activity of this abundant nuclear protein (Ludwig et al. 1988; Yamanaka et al. 1988). Therefore associative properties of ADPRT may play a more important physiological function and the trace polymerase activity could be regarded as a probably self-regulatory mechanism that may control macromolecular associations of ADPRT (Zahradka and Ebisuzaki 1982). Macromolecular self-association of ADPRT molecules actually control the polymerase activity of this protein and in concentrations of ADPRT present in nuclei self-association inhibits the polymerase (Bauer et al. 1990), explaining the observed low cellular enzymatic activity (Yamanaka et al. 1988).

The participation of histones in poly (ADP-ribosylation) has been amply documented experimentally (cf. Ueda and Hayaishi 1985; Althause and Richter 1987), yet the exact nature of the role of histones in the poly (ADP-ribosylation) process remained obscure. For example it was unexplained why the ADPRT-catalyzed reaction is accelerated by histones (Hakam et al. 1988). The association of poly ADP-ribosylated nucleosomes with active and inactive regions of the chromatin of HeLa cells (Wong and Smulson 1984) and a relationship between hyperacetylation of histones H3 and H4 and poly ADP-ribosylation of histone H1 has been extensively studied (Hough and Smulson 1984) and these results suggest a

probable cell biological regulatory role of histone-ADPRT association that requires further elucidation. A direct binding of purified ADPRT to histones was demonstrated with the nitrocellulose filter binding assay (Sastry and Kun 1988). However an ordered addition requiring cooperative binding of histone-DNA or ADPRT-DNA complexes with either ADPRT or histones respectively (Sastry and Kun 1988) indicated that DNA plays an important, but unexplained, role in macromolecular association involving ADPRT.

We demonstrate in this paper that a histone-Sepharose 4B affinity matrix binds ADPRT in a strictly DNA-dependent manner and among the polypeptides of ADPRT obtained by digestion with plasmin (Buki and Kun 1988) only the terminal 29 kDa polypeptide associates with the histone affinity matrices, requiring also DNA for binding. We also show that the 29 kDa polypeptide competitively inhibits the binding of histones and DNA to ADPRT. Since besides histones, polyamines represent physiologically important multifunctional polycations (Tabor and Tabor 1976, 1984) and it has been shown that polyamines influence poly ADP-ribosylation (Perella and Lea 1979; Melvin and Keir 1979; Wallace et al. 1984) we extended our experiments to include spermine and synthetic poly amino acid analogs and determined both ADPRT binding properties and their effect on the polymerization reaction.

Materials and Methods

More than 95% homogeneous calf thymus ADPRT was isolated as reported (Buki et al. 1987). Coenzymic DNA as a byproduct of enzyme isolation was further purified by phenol extraction and ethanol precipitation and consisted mainly of 2-4 kb double-stranded DNA fragments (Buki et al. 1987). The coenzymic synthetic octadeoxyribonucleotide duplex (octamer C) was synthesized as described (Hakam et al. 1987). Polyclonal rabbit-anti-ADPRT IgG was prepared as published (Buki et al. 1987). The silver-enhanced immunogold system was purchased from Janssen (Piscataway, NJ) and histones from Sigma (St. Louis, MO). BrCN-Sepharose 4B was a product of Pharmacia (Piscataway, NJ) and [32 P]-NAD of ICN (Irvine, CA). Other methods were the same as published earlier (Bauer et al. 1990; Buki and Kun 1988).

Immobilized histones were prepared from CNBr-activated Sepharose 4B gel by first washing the gel (0.5 ml aliquots) with 1 mM HCl. The gel was then suction-dried and incubated with 4 mg aliquots of histone H1, H2A, H2B, H3, or whole histones, dissolved in 0.1 M NaHCO₃ at pH 8.4 + 0.5 M NaCl for 6 hours at 4°C, then 200 mM (final conc.) ethanolamine (pH 8.4) was added and incubation continued overnight. The resins were then washed successively with 10 ml of 0.2 M KH₂PO₄ (pH 4.25), 0.5 M NaCl; 10 ml of H₂O; 10 ml of 0.2 M Tris-HCl (pH 9.0), 0.5 M NaCl, and finally with, and suspended in, the binding buffer (25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.2 mM DTT and 0.1 M NaCl). The amount of histones bound to the Sepharose 4B resin was determined in 50- μ l aliquots of the settled resin by staining with Coomassie Brilliant Blue

and destaining in 10% acetic acid. The amount of protein-bound dye was determined colorimetrically after elution of the dye as described (Buki et al. 1987). For calibration, histones were subjected to electrophoresis according to Panyim and Chalkley (1969) and the gels stained and destained and the protein-bound dye eluted and quantitated as reported (Buki and Kun 1988). On the average 0.9-1.2 mg of histones were covalently bound per ml of Sepharose 4B sedimented at 200 x g for 5 min. Preparation of polylysine- (10.2 kDa), polyarginine- (11.6 kDa), and spermine-Sepharose 4B was carried out as described for histone-Sepharose. The polylysine, polyarginine and spermine content of the matrix was 0.75 mg, 1.2 mg and 0.4 mg per ml resin respectively, as determined by colorimetry (Fields 1972).

Binding assay of ADPRT to whole histone-Sepharose 4B.

Fifty ul of sedimented Sepharose 4B as control or whole histone-Sepharose 4B were incubated with 4 ug of ADPRT in the absence or presence of coenzymic DNA (135 ug/ ml) in a total volume of 100 ul for 10 minutes at room temperature. At the end of the incubation the suspensions were centrifuged and aliquots (25 ul) of the supernatants were mixed with equal volumes of double-strength sample buffer and loaded onto 10% SDS-PAGE gels, representing the unbound fraction of the enzyme. The pelleted resin was washed twice with 0.5 ml aliquots of the binding buffer by resuspension and centrifugation, and the resin-bound ADPRT was eluted with 50 ul of the binding buffer containing 1 M NaCl, by incubation for 20 minutes at room temperature with occasional shaking. After centrifugation, 25-ul aliquots of the superna-

tants mixed with an equal volume of sample buffer were loaded onto 10% SDS-PAGE gels, representing the bound enzyme fraction. Gel electrophoresis and Coomassie Brilliant Blue gel staining and destaining were performed as published (Bauer et al. 1990).

Binding of ADPRT and polypeptide fragments of ADPRT to various histone subtypes and to polylysine-, polyarginine-, and spermine-Sephrose 4B resins

Four ug of ADPRT or aliquots of proteolytic digests of 5 ug ADPRT were incubated with various Sepharose 4B resins (25 ul) in a total volume of 50 ul. The incubations and other manipulations were the same as described earlier for ADPRT with the following modifications. After electrophoresis the polypeptides were transblotted onto nitrocellulose sheets (Buki et al. 1987) and visualized by the silver-enhanced immunogold staining technique.

Poly(ADP-ribose) polymerase assays were carried out by published techniques (Buki and Kun 1988) with modifications as given in the legends.

Nucleosome reconstruction: (Stein 1987) 600 ug coDNA and an equal amount of core histones (Sigma, Type II-AS) were dissolved in 1.5 ml of TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) containing 0.8 M NaCl. This solution was dialyzed (at 4°C) stepwise against TE buffer that contained progressively lower concentrations of NaCl (0.8, 0.6, 0.4 and 0.2 M NaCl). Each dialysis lasted 90 minutes except the final dialysis against TE buffer containing no added NaCl was extended overnight. The DNA content was adjusted to 200 ug/ml by dilution.

Analysis of auto-ADP-ribosylation of ADPRT and ADP-ribosylation of histones by acidic urea-SDS-PAGE (Jackowski and Kun 1983). ADPRT (1.2 ug) was preincubated for 20 minutes at 23 °C either with coDNA (10 ug), with or without histones in solution, or alternatively with reconstructed nucleosomes, containing both DNA and histones at the same concentration as present in the soluble system in 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM DTT and 10 mM MgCl₂ in a final volume of 100 ul. The polymerase reaction was started by the addition of ³²P-NAD (200 uM NAD, 10 cpm/nmol) and the reaction (at 23 °C) was terminated by precipitation with cold TCA (final concentration 20%) and filtration onto Whatman GF/C filters. The filters were extensively washed with 20% cold TCA (5 x 5 ml), a procedure necessary to remove occasional contaminants of commercial [³²P]-NAD which on a gel migrate in a position simulating 20-40 kDa. The proteins were then extracted at 4 °C from the filters with 500 ul of sample buffer per cm² filter and separated by the urea-SDS-PAGE system (Jackowski and Kun 1983) and the gel electrophoresis and autoradiography performed as reported (Buki et al. 1987) with a 50 ul aliquot. More than 85% of radioactive material present in TCA precipitates on the filters was extracted by the above technique.

Results

The binding of ADPRT to the histone-Sepharose 4B matrix had an absolute requirement for coenzymic DNA as shown in Fig. 1. The unmodified Sepharose 4B had no binding capacity, thus the histone-Sepharose 4B exhibited the characteristics of a histone-specific

affinity matrix. Results obtained by this technique appeared to be at variance with the demonstration of a direct histone-ADPRT binding, as detected by the nitrocellulose filter binding assay (Sastry and Kun 1988). The inherent difficulty of interpretation of both tests is their dependence on the strengths of macromolecular associations. The filter binding assay may detect relatively weak associations whereas retention on the affinity matrix may depend on much stronger macromolecular binding forces which is also suggested by the previously observed cooperative binding that was dependent on an ordered addition of macromolecules (Sastry and Kun 1988). Although clarification of this problem will require techniques that can determine binding constants, at this stage it seems reasonable to assume a relatively strong DNA-dependent association of ADPRT to the histone-matrices.

A similar DNA-dependent and selective binding of the 29 kDa terminal basic polypeptide of ADPRT to the histone-affinity matrices was demonstrated in Fig.2. As in Fig.1, panel A illustrates the gel analysis of flow-through polypeptides, where the missing bands (lanes 4, 6 and 10) correspond to polypeptides bound to the matrix in the presence of DNA. No particular selectivity towards histones H1-, H2A-, H2B- or H3-matrices could be demonstrated by this method, except polypeptide specificity of binding was confined to the 29 kDa polypeptide of ADPRT. Panel B is a mirror image of A and illustrates the gel separation of matrix-bound polypeptides that were eluted by 1M NaCl, the main polypeptide being the 29 kDa species. Weak bands of the other polypeptides

indicates incomplete washout of non-specifically retained trace polypeptides. Coenzymic DNA could be replaced by 20 ug/ml of a synthetic octadeoxyribonucleotide (duplex C, cf. Hakam et. al. 1987) (results not shown).

The binding of ADPRT and its polypeptides, prepared by digestion with plasmin, was also tested with Sepharose 4B matrices containing covalently bound polylysine, polyarginine and spermine as affinity ligands, to be compared with the histone-Sepharose 4B binding system. Since the experimental design was the same as employed in tests illustrated in Figs. 1 and 2, instead of repetitious gel analysis a semiquantitative assessment of the binding of polypeptides to affinity matrices was developed that was based on the colorimetric estimation of bound and free polypeptides separated in gels (Buki and Kun 1988). We defined strong binding when at least 50% of the polypeptides were retained on the affinity matrix whereas retention between 10 and 40% was defined as weak and less than 10% as very weak. In the experiments illustrated in Figs. 1 and 2 histone-Sepharose 4B strongly bound both ADPRT and the 29 kDa polypeptide, in the presence of either the Co-DNA or the synthetic octamer C. The behaviour of the polylysine-Sepharose 4B matrix was similar to that of the histone matrix, except only ADPRT bound strongly and the 29 kDa polypeptide associated only weakly in the presence of either of the two DNA coenzymes. Spermine-Sepharose 4B bound ADPRT and the 29 kDa polypeptide only very weakly and only in the presence of coDNA, and not at all in the presence of octamer C, whereas polyarginine-Sepharose 4B by itself strongly bound

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both the 56 kDa polypeptide and ADPRT in the absence of DNA, but the weak binding of the 29 kDa polypeptide required either of the two DNA coenzymes. According to these results certain similarities exist between histone, polylysine, polyarginine and spermine binding to ADPRT, except that polyarginine can bind ADPRT even without DNA at the 56 kDa polypeptide domain.

If the DNA-dependent binding of polycations to the 29 kDa terminal polypeptide has a meaningful regulatory function on rates of poly ADP-ribosylations, then the certain externally added polypeptides of ADPRT to a catalytic system should inhibit the enzymatic process by competing with either histones or DNA or both. Because of the scarcity of polypeptides obtained by plasmin digestion, kinetic assays were confined to two types of limiting conditions: first, when the rate of ADPRT catalyzed reaction was limited by the concentration of the coenzymic DNA analog (octamer C), and second, when V_{max} was dependent on the concentration of the activating histone H3. In these enzyme kinetic tests we preferred to use octamer C to coDNA, because the former is a well defined chemical entity (Hakam et al. 1987) and as shown in Fig. 5A there is a large activation by histones with the octamer as coenzyme. Results are summarized in the Table. Without any addition or with histone H3 alone only trace enzymatic activity was detected (Nos. 1,2) which was significantly augmented by non-saturating (No. 3) or saturating concentrations (No.8) of octamer C. At non-saturating concentrations of octamer C, histone H3 added at saturating concentration greatly augmented enzymatic activity (No. 4). An addition of the 29 kDa (histone-binding)

polypeptide to this system significantly inhibited ADPRT activity (No. 5). The 36 kDa polypeptide (No. 6) had a weaker inhibitory action than 29 kDa polypeptide, and the 56 kDa polypeptide which did not bind to the histone- or the DNA-matrix (Buki and Kun, 1988) (No.7), had no significant inhibitory effect. When saturating concentrations of octamer C (No. 8) but non-saturating concentrations of histone H3 were present (Nos. 9 to 12) the 29 kDa polypeptide inhibited enzymatic activity by ca. 50% (No. 9) but the other polypeptides had no detectable influence on enzymatic rates (Nos. 11,12), suggesting that the histone-binding polypeptide was an effective competitive inhibitor when either octamer C or histone H3 were limiting ADPRT activity. When octamer C and histone H3 were added both at saturating concentrations (No.13) the apparent V_{max} of ADPRT was obtained, which was not altered by the addition of polypeptides at concentrations which were inhibitory under above limiting kinetic conditions, indicating the predicted competition at the histone and/or DNA binding sites.

A Dixon-type kinetic analysis of the two limiting conditions is shown in Fig. 3. In the top panel (A), when histone H3 is limiting (56 nM, top curve), the 29 kDa polypeptide acted as a competitive inhibitor. At higher histone H3 concentrations (280 to 1900 nM, the second and third curves, respectively) the inhibitory effect of the 29 kDa polypeptide diminished as would be predicted.

At rate-limiting concentrations of the octamer C (11.3 nM, top curve of panel B) the 29 kDa polypeptide also behaved as a

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competitive inhibitor with respect to the octamer and this effect diminished when octamer C concentration was raised (45 nM or 300 nM, curves 2 and 3 of panel B, respectively).

At limiting octamer but saturating H3 concentrations the 36 kDa polypeptide exhibited a competitive inhibition vs. the octameric duplex coenzyme (Fig. 4) as would be expected, since this 36 kDa polypeptide bound to a DNA affinity matrix (Buki and Kun, 1988). The calculated binding constant at 20 nM ADPRT concentration for the octamer duplex C was $K_D = 15$ nM, whereas K_I vs octamer C for the 29 kDa polypeptide was 0.9 μ M and K_I vs octamer C for the 36 kDa polypeptide was 1.6 μ M. Since K_D and K_I have the same dimensions it is possible to compare them. The proteolytic cleavage of the histone- and DNA-binding (Buki and Kun 1988) domain of ADPRT into 29 and 36 kDa polypeptides greatly decreased their affinity towards the octamer C binding site by 60-fold (from 15 nM to 0.9 μ M). On the other hand the K_D of histone H3 in the histone - ADPRT - octamer complex (0.7 μ M) and K_I for the 29 kDa polypeptide towards histone in the ADPRT - octamer - histone complex (1.5 μ M), remained in the same order of magnitude, suggesting that the specificity of the 29 kDa polypeptide as a histone binding-site oriented inhibitor was closely retained after its proteolytic splitting from the ADPRT molecule.

The regulatory effects of polycations on enzymatic rates of self-ADP-ribosylation of ADPRT was further investigated. As shown in Fig. 5 all four polycations exerted characteristic catalytic

effects on initial rates of auto-poly ADP-ribosylation of ADPRT. Panel A in Fig. 5 illustrates that increasing concentrations of histones (mixed histones or purified histone fractions) greatly increased initial rates (V_{\max} read at 4 min) of poly ADP-ribosylation of ADPRT when octamer C was the coenzyme and much less in the presence of coDNA. On the other hand as shown in panel B, spermine at concentrations known to exist in animal cells (e.g. in rat prostate, cf. Ahmed et al. 1985) proved to be the most potent enzyme activator when the probably more physiologically meaningful co-DNA served as the ADPRT coenzyme (panel B of Fig. 5). The preference towards coDNA agrees with the coDNA requirement of ADPRT binding to the spermine-Sepharose matrix. Polylysine only moderately activated initial rates and did not discriminate between the two types of DNA coenzymes (panel C, Fig. 4). At relatively low concentrations, polyarginine in the presence of coDNA (up to about 2.5 μ M) activated ADPRT, approximating the effect of histones, except beyond this concentration polyarginine depressed rates, i.e. the effect appeared biphasic. There was only marginal activation by polyarginine when octamer C served as a coenzyme (panel D, Fig. 5).

Under the experimental conditions described in Fig. 5 only ADPRT was poly ADP-ribosylated as assayed by urea-SDS-PAGE (Jackowski and Kun 1983). Therefore the increase in reaction rates could not be explained by poly ADP-ribosylation of the polycations serving as secondary acceptors (gel results not shown). However this important point was further analyzed with histones as activating polycations. The absence of histone poly ADP-

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ribosylation by ADPRT in solution with the purified enzyme has been observed also by others (Ikeyima et al. 1987). A slow rate of Schiff base formation between ADPR and polycations at basic pH is possible (Kun et al. 1976) and subsequent elongation of these adducts by ADPRT has been reported (Ueda et al. 1979). However the short reaction time employed in our experiments excludes this reaction sequence. On the other hand the well known nuclear poly ADP-ribosylation of histones (cf. Ueda and Hayaishi 1985; Althaus and Richter 1987), the immunochemical identification of larger than tetrameric poly ADP-ribose moieties covalently bound to histones in vivo (Minaga et al. 1979) and the large number of ADP-ribosylated histones identified in dimethylsulfate-poisoned myeloma cells (Boulikas 1988) required an explanation. As illustrated in Fig. 6 histones incorporated into reconstituted nucleosomes were readily poly ADP-ribosylated by highly purified ADPRT (lane 3 of Fig. 6) in vitro, whereas in a soluble, nonstructured system only ADPRT was auto-poly ADP-ribosylated in the presence of coDNA and histones (Fig. 5, lanes 1 and 2) even though the same components were present from which nucleosomes have been reconstructed (see Materials and Methods), suggesting a highly complex supramolecular regulation of histone poly ADP-ribosylation, which occurs only after nucleosome assembly.

In a soluble system the protein concentration-dependent self association of ADPRT molecules regulates rates of poly ADP-ribosylation (Bauer et al. 1990). We tested the effect of histones on the decrease of polymerase activity of ADPRT, which occurs at enzyme concentrations below 20 nM and is presumably due

to the dissociation of dimers to monomers (Bauer et al. 1990). As illustrated in Fig 7, histones prevented the dilution-dependent decrease of poly ADP-ribose polymerase activity of ADPRT indicating that in the presence of octamer C histones appear to stabilize the ADPRT dimer. As the monomers of ADPRT exhibited also an increased NAD-glycohydrolase activity (Bauer et al. 1990) we tested the effect of histones and DNA on the NAD-glycohydrolase activity of ADPRT and found that both histones and DNA activate the hydrolytic activity of ADPRT towards NAD, coincidental with the self-association regulated polymerase activity (Kirsten et al. unpublished results).

Discussion

Although significant advances were made in the elucidation of the primary structure of ADPRT by cDNA sequencing in several laboratories (e.g. Kurosaki et al. 1987, Cherney et al. 1987) as well as by the analysis of the structure of the human ADPRT gene (Auer et al. 1989), the relative constancy of ADPRT molecules/cell in cell cultures, exhibiting varying biological behavior (Ludwig et al. 1988, Yamanaka et al. 1988, Kirsten et al. 1990, unpublished), tends to suggest that the main cell biochemical function of ADPRT may largely depend on the intracellular catalytic and associative activities of this "housekeeping" protein.

The identification of the 29 kDa and 36 kDa polypeptides of ADPRT possessing DNA-binding ability (Buki and Kun, 1988) and the localization of two Zn^{2+} finger motifs in the 29 kDa terminal

polypeptide (Mazen et al. 1989, de Murcia et al. 1989, Gradwohl et al. 1990) representing the binding sites for DNA termini, that are required for the coenzymic function of DNA in poly ADP-ribosylation, while pinpointing important binding sites still fall short of providing a molecular mechanism of the ADPR transferase reaction, which is now further complicated by the probability that two molecules of ADPRT may actually be required for the catalysis (Bauer et al. 1990). Our results show that the histone binding site containing 29 kDa polypeptide also competes with DNA binding to ADPRT, which is consistent with the coexistence of both the DNA terminus-recognizing and histone binding sites in the 29 kDa polypeptide. The significant (60-fold) decrease in the affinity of the 29 kDa and 36 kDa polypeptides towards DNA, as compared to the intact ADPRT protein, in contrast to the unchanged affinity towards histones exhibited by the 29 kDa polypeptide, supports the idea that both histone and DNA binding sites reside in the 29 kDa polypeptide and that these binding domains of the 29 kDa polypeptide represent an important polycation-specific regulatory site of enzymatic poly ADP-ribosylation. Furthermore the fact that the 36 kDa polypeptide can also compete with a catalytically activating DNA binding reaction of ADPRT (Fig. 4) supports the existence of a second DNA binding domain in the ADPRT molecule in addition to the DNA terminus-specific Zn^{2+} finger polypeptide sites located on the 29 kDa terminal polypeptide. However the participation of the 36 kDa basic polypeptide in DNA binding is presently less defined than the DNA binding sites for DNA termini on the 29 kDa polypeptide. Since the 36 kDa peptide can replace

the ADPRT molecule in DNase I protection tests at internal regions of certain dsDNAs (Sastry and Kun, 1989) we assumed that these internal ADPRT binding sites on certain dsDNAs may be important in the topological action of ADPRT on DNA conformations (Sastry and Kun 1988). The chain elongation of protein-bound oligomers was shown to be greatly activated by coDNA (Bauer and Kun 1985) implying that both the initiation and elongation processes of poly ADP-ribosylation require DNA, thus there is an opportunity for two binding sites for DNA, one for initiation and one for elongation of poly ADP-ribose. Details of these processes are still missing.

The unusual DNA selectivity of the two physiologically occurring polycations as activators of the poly ADP-ribosylation reaction (Fig. 5) are not readily explained by the assumption that only the DNA termini are important as significant ADPRT binding sites. The activating effect of histones is much greater when octamer C is the coenzyme as compared to coDNA (Fig. 5A), whereas exactly the opposite is the case when spermine is the polycation (Fig. 5B). In neither case can the rate increase be explained by an acceptor role of polycations. If only the number of DNA termini regulates V_{\max} of the poly ADP-ribosylation reaction then the octamer should be the most effective coenzyme, regardless of the polycation present, therefore the unexpected fastidiousness of the enzyme towards the DNA analogs used as coenzymes suggests that the maximal activation by spermine, that depends on coDNA, may involve DNA structures which are present in coDNA but are absent in octamer C. The coincidence or

)

vicinity of the binding sites of DNA and polycations on the 29 kDa polypeptide appears to have basic consequences in the physiological regulation of poly ADP-ribosylation. Two aspects of this problem may be analyzed.

The role of histones as activators depends on the superstructure of the histone-DNA-ADPRT system. In solutions histones act only as polycationic activators, but once a nucleosome structure is formed histones are vigorously poly ADP-ribosylated, thus they are also ADPR-sinks and a structural regulation of chromatomers by histone ADP-ribosylation becomes possible. This duality may have biological importance since it is known that at the end of the cell cycle half of the histones are newly synthesized and are present in various histone pools to form nucleosomes (Tsvetkov et al. 1989), thus real opportunities for both types of effects of histones on poly ADP-ribosylation rates exist.

Spermine is the most powerful activating polycation of ADPRT at physiological concentrations when applied as the single polycation (Fig. 4B), but can exert also an inhibitory function on the activation by histones at certain histone concentrations (results not shown). Interference with polyamine metabolism by enzyme site-oriented drugs of polyamine pathways produces alterations in various cell functions that are likely to be related to changes in gene regulations (McCann et al. 1987). Since our results provide evidence for the participation of spermine in an ADPRT-mediated regulatory system that can modify DNA structures, it seems plausible to assume that ADPRT may play

a macromolecular role in the mode of action of drugs that alter polyamine metabolism, especially since it is now known that ADPRT is probably the most abundant DNA-binding protein of eukaryotes (Ludwig et al. 1988, Yamanaka et al. 1988). Artificial analogs of histones, such as polylysine and polyarginine, mimic to some extent the effect of both physiological polycations, but cannot fully replace them.

Acknowledgments

This work was supported by a grant of the Air Force Office of Scientific Research (AFOSR-89-0231). The contribution of the Drs. Eva Kirsten and Jerome Mendeleyev to the preparation of the manuscript is highly appreciated.

Abbreviations

CNBr, cyanogen bromide; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; CoDNA, coenzymic DNA; Octamer C, synthetic octadeoxyribonucleotide duplex C (Hakam et al. 1987).

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Legend to Fig. 1.

Binding of ADPRT to histone-Sepharose 4B resins.

Four ug of ADPRT were incubated either with Sepharose 4B or with histone-Sepharose 4B resins in the presence or in the absence of coenzymic DNA (Materials and Methods). Panel A (lanes 1-4) shows the unbound ADPRT that flows through the column and panel B (lanes 1-4) the resin-bound ADPRT that was eluted from the resin with the binding buffer containing 1M NaCl. Lane 1: Sepharose 4B incubated with ADPRT in the absence of coenzymic DNA. Lane 2: Sepharose 4B incubated with ADPRT in the presence of coenzymic DNA. Lane 3: Histone-Sepharose 4B incubated with ADPRT in the absence of coenzymic DNA. Lane 4: Histone-Sepharose 4B incubated with ADPRT in the presence of coenzymic DNA. The far right lane on panel B illustrates the molecular weight markers (myosin, β -galactosidase, phosphorylase B, BSA, ovalbumine and carbonic anhydrase).

Legend to Fig.2.

Binding of polypeptide fragments of ADPRT obtained by plasmin digestion to various histone subtypes covalently bound to Sepharose 4B resins.

For each lane, 5ug of plasmin-digested ADPRT was incubated with various histone-Sepharose 4B resins (Materials and Methods). Panel A shows the immunogold-stained Western blot of the flow through fractions, panel B the immunoblot of the peptide patterns of matrix-bound fractions eluted by 1M NaCl. Lanes 1&2 are

controls: Sepharose 4B without (1) and with (2) coenzymic DNA. Lanes 3&4: Histone-H1-Sepharose 4B without (3) and with (4) coenzymic DNA. Lanes 5 & 6: Histone-2A-Sepharose 4B without (5) and with (6) coenzymic DNA. Lanes 7 & 8: Histone-H2B-Sepharose 4B without (7) and with (8) coenzymic DNA. Lanes 9 & 10: Histone-H3-Sepharose 4B without (9) and with (10) coenzymic DNA. Lane 11: peptides of ADPRT obtained by digestion with plasmin. On the far right of each panel the molecular weights of the four main peptides obtained by plasmin digestion of ADPRT are illustrated. Lanes marked with arrows are those where specific binding to the resins was observed in the presence of DNA.

Legend to Fig. 3.

Inhibition of ADPRT activity by the 29 kDa polypeptide:
competition with histone-H3 (A) and with octamer C (B).

ADPRT (20 nM) was preincubated with various concentrations of 29 kDa polypeptide (see abscissa) either in the presence of a constant concentration of octameric DNA and various concentrations of histone-H3 (panel A, 300 nM of octamer C and: +—+, 56 nM H3; ○—○, 280 nM H3; ■—■, 1900 nM H3) or in the presence of a constant concentration of H3 and various concentrations of octamer C (panel B, 14 uM of H3 and: +—+, 11.3 nM octamer C; ○—○, 45 nM octamer C; ■—■, 300 nM octamer C) for two minutes at 23°C. The volume of the reaction mixture was 100 ul and it consisted of 50 mM Tris-HCl buffer (pH 8.0), 100mM NaCl, 10 mM MgCl₂ and 2mM DTT. The reaction was started by adding

[^{32}P]-NAD to the reaction mixture (200 μM final conc., 10^4 cpm/nmol). Two-minute rates were measured and the enzymic reaction was stopped by adding 2ml of 20% TCA to the reaction mixture. The samples were filtered onto Whatman GF/C filters, washed with 4x5 ml of 20% TCA and with 2x2ml of ethanol and then dried. The amount of incorporated ADPR was determined by liquid scintillation spectrometry.

Legend to Fig. 4.

Inhibition of ADPRT activity by the 36 kDa polypeptide, competition with the octadeoxyribonucleotide duplex (octamer C).

ADPRT (20 nM) preincubated with varying concentrations of 36 kDa polypeptide shown in the abscissa in the presence of constant concentration of histone H3 (14 μM) and various concentrations of octamer C (\bigcirc — \bigcirc = 45 nM octamer C, \square — \square = 11.3 nM octamer C and $+—+$ = 300 nM octamer C) for two minutes at 23 $^{\circ}\text{C}$. The assay of ADPRT activity was carried out as described in the legend of Fig. 3.

Legend to Fig. 5.

Effect of polycations on the ADPRT activity.

ADPRT (20 nM) was preincubated with histones (panel A), spermine (panel B), polylysine (panel C), and polyarginine (panel D) for 12 minutes either in the presence of octamer C (400 nM) open symbols or in the presence of coDNA (200 $\mu\text{g/ml}$) closed symbols at 23 $^{\circ}\text{C}$. The

reaction mixture contained 100 mM Tris-HCl pH 8.0 buffer and 10 mM of 2-mercaptoethanol. The reaction was started by adding [32 P]-NAD (200 uM final NAD concentration, 10^4 cpm/nmol) to the incubation mixture. Four-minute rates were measured and the amount of incorporated ADPR was determined by liquid scintillation spectrometry.

Legend to Fig. 6.

Poly ADP-ribosylation of ADPRT and histones in reconstructed nucleosome (28).

Experimental conditions are detailed in Materials and Methods.

Lane 1, ADPRT + coDNA; Lane 2, ADPRT + coDNA + histones;

Lane 3, ADPRT + reconstructed nucleosome.

Legend to Fig. 7.

Effect of ADPRT concentration on the specific activity of the enzyme in the absence and presence of histone H3.

ADPRT (2.4 μ g) was incubated in the standard reaction mixture either in the absence ($\square-\square$) or in the presence ($\circ-\circ$) of 200 ug/ml histone H3 for one minute at 23 °C. The volumes of the reaction mixtures were varied between 10-2000 μ l. To achieve lower ADPRT concentrations the ADPRT stock was ten-fold diluted. The reactions were started by adding one ul of ADPRT to the mixture. The specific activities of ADPRT are plotted against ADPRT concentrations used in the experiments.

Table

TABLE

Inhibition of ADPRT activity by polypeptides of ADPRT

No.	Experimental Condition	ADPRT activity ^a (pmol ADPR/pmol ADPRT x min)
1	ADPRT	0.12 <i>new data</i>
2	ADPRT + histone H3 (200 ug/ml)	0.12 <i>new experiment</i>
3	ADPRT + octamer C (0.075 ug/ml)	3.72
4 ^b	ADPRT + octamer C (0.075 ug/ml) + H3 (200 ug/ml)	38.70
5 ^b	No. 4 + 29 kDa peptide (10 ug/ml)	12.40
6 ^b	No. 4 + 36 kDa peptide (10 ug/ml)	23.50
7 ^b	No. 4 + 56 kDa peptide (12 ug/ml)	37.20
8	ADPRT + octamer C (2 ug/ml)	12.50
9 ^c	ADPRT + octamer C (2 ug/ml) + H3 (0.8 ug/ml)	36.00
10 ^c	No. 9 + 29 kDa peptide (10 ug/ml)	19.80
11 ^c	No. 9 + 36 kDa peptide (10 ug/ml)	33.70
12 ^c	No. 9 + 56 kDa peptide (12 ug/ml)	36.70
13	ADPRT + octamer C (2 ug/ml) + H3 (200 ug/ml)	101.70

^a The ADPRT activity was assayed as described in the legend of Fig. 3, with the exception that 0.1 ug of ADPRT was used in these experiments.

^b Experiments where the enzyme activity is limited by the concentration of octamer C.

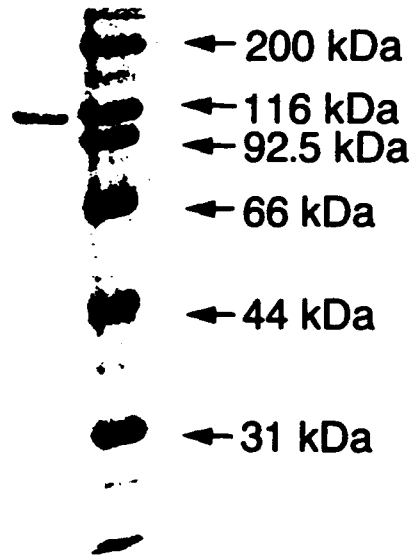
^c Experiments where the enzyme activity is limited by the concentration of histone H3.

New results. ...

Fig. 1

A

B



1 2 3 4 1 2 3 4

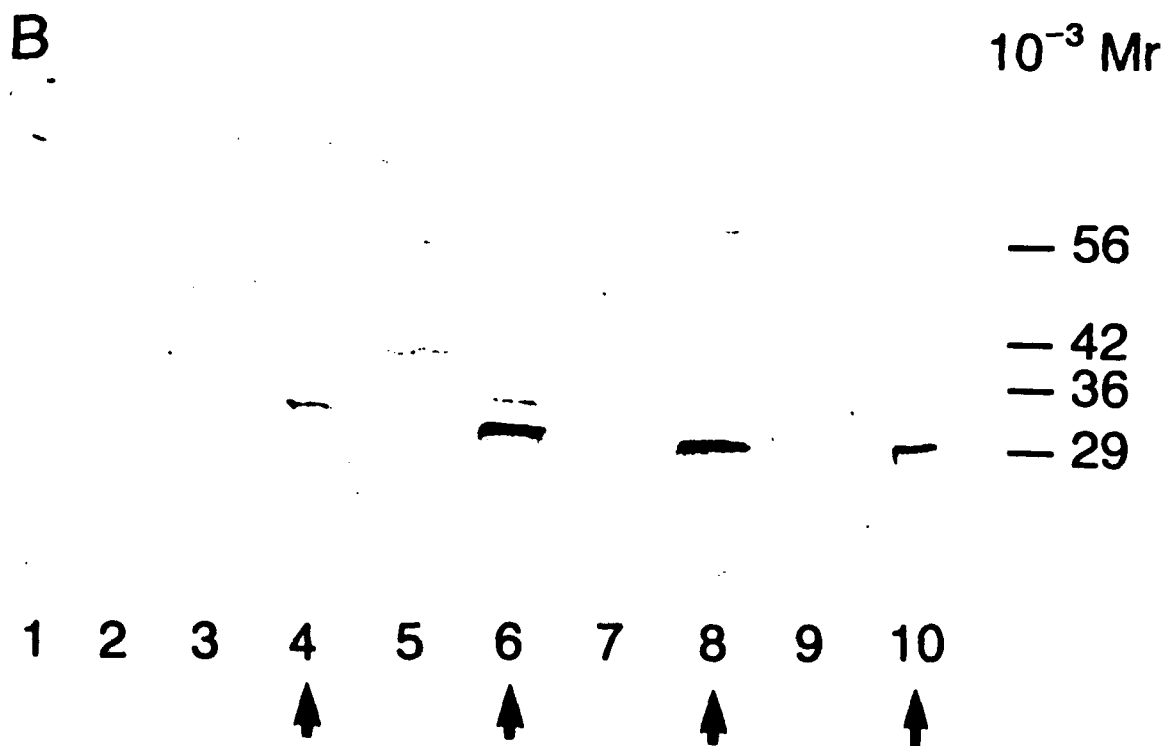
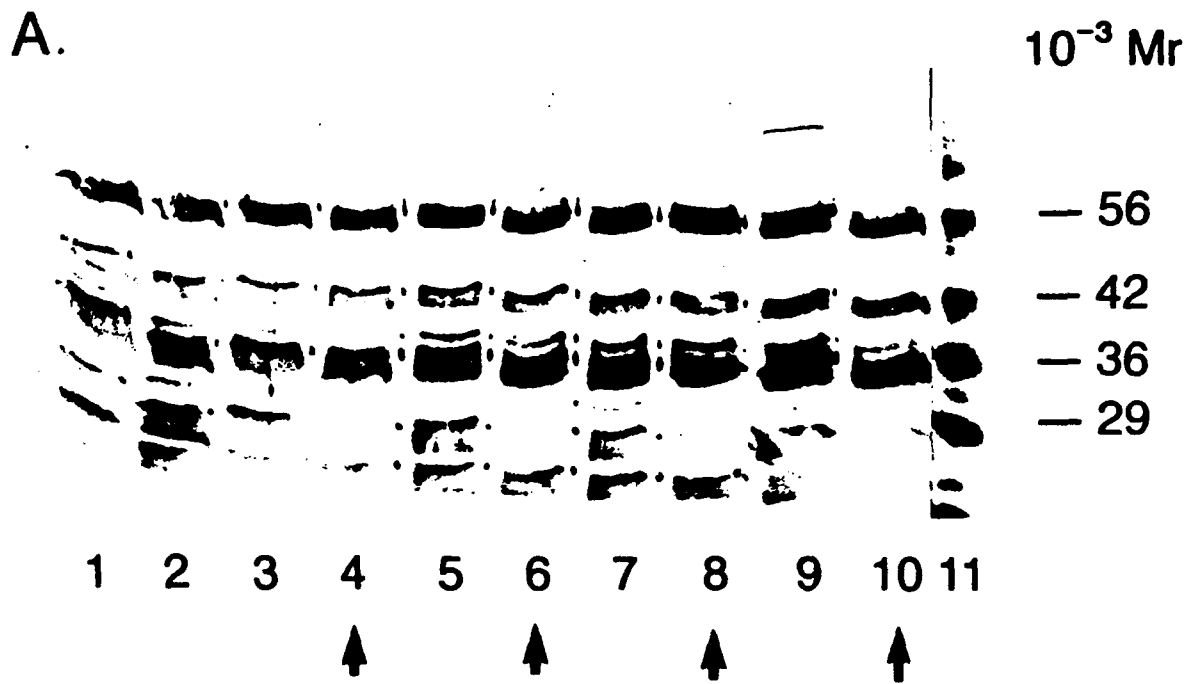


Fig. 3

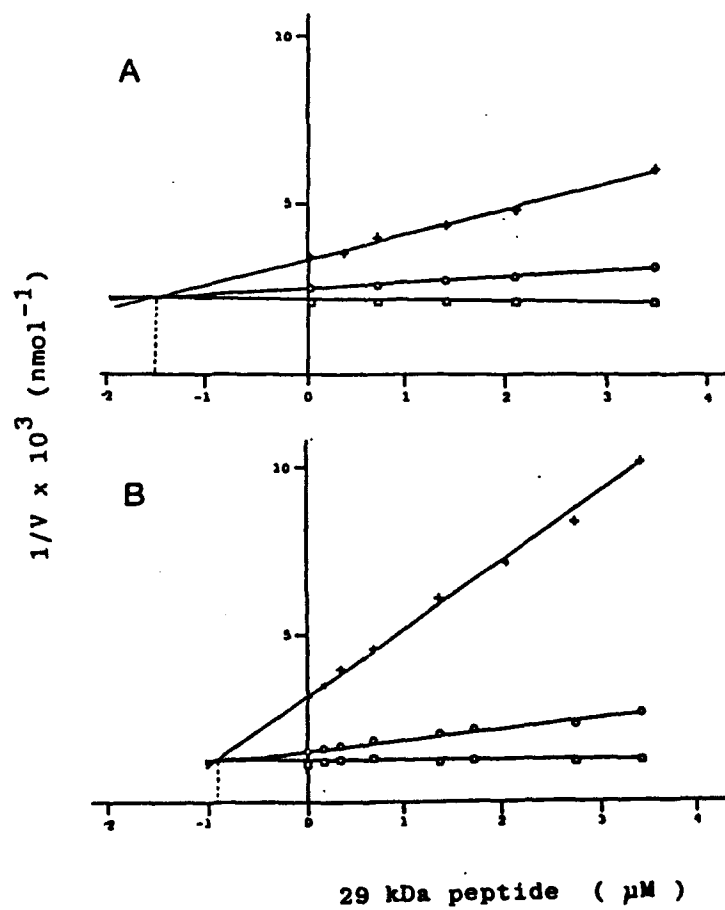


Fig. 11

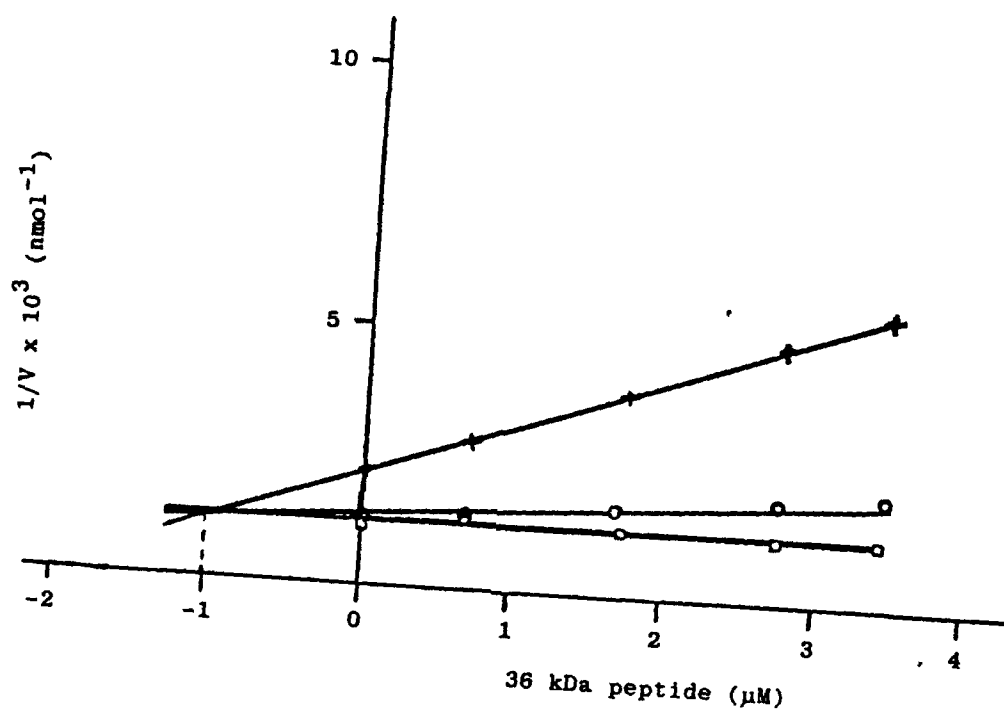


Fig 5

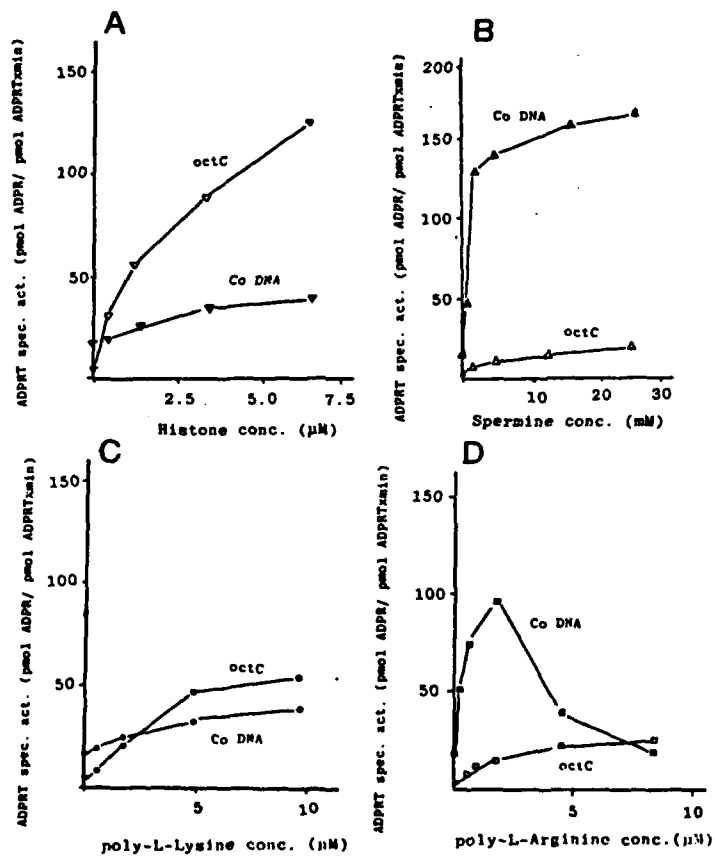


Fig. 6



Mr (kDa)

— 200

— 116

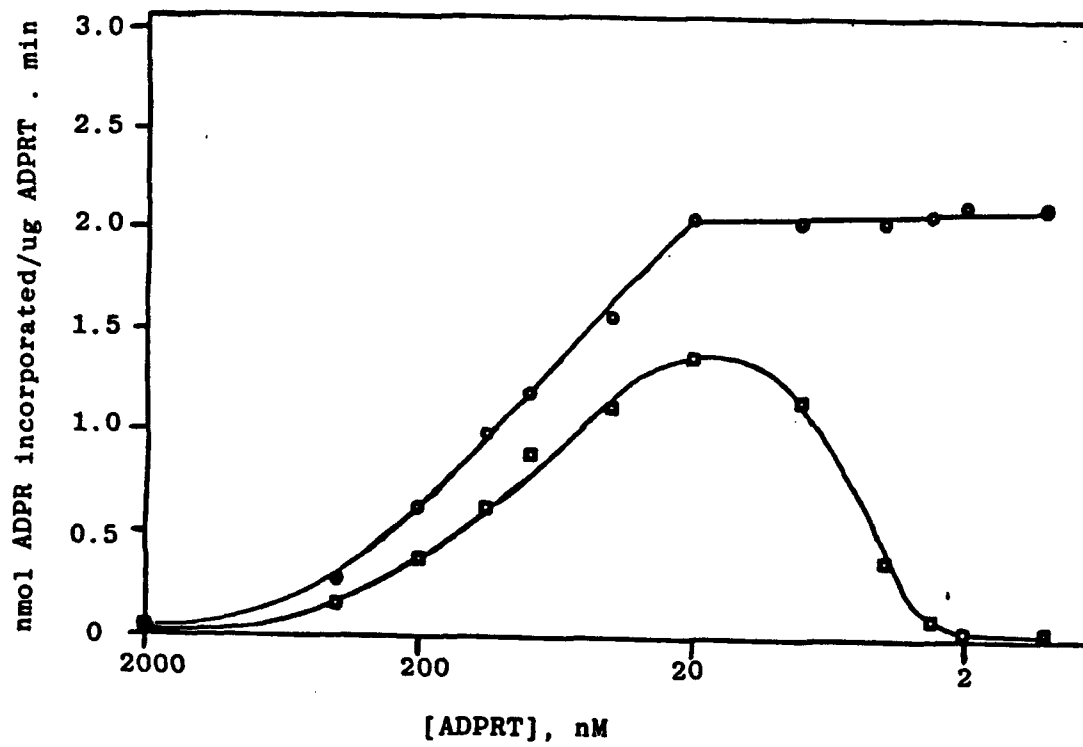
— 98

1

2

3

Fig. 7



II

Destabilization of Zn(II) Coordination in ADP-Ribose Transferase (Polymerizing) by 6-Nitroso-1,2-benzopyrone Coincidental with Inactivation of the Polymerase but not the DNA Binding Function.

Summary

6-nitroso-1,2-benzopyrone, an oxidation product of 6-amino-1,2-benzopyrone, binds to the DNA-recognizing domain of the ADP-ribose transferase protein and preferentially destabilizes Zn^{2+} from one of the two zinc finger polypeptide complexes present in the intact enzyme, as determined by the loss of 50% of $^{65}\text{Zn}^{2+}$ from the $^{65}\text{Zn}^{2+}$ -loaded protein molecule, coincidental with the loss of 99% of enzymatic activity. The 50% zinc-deficient enzyme still binds to a DNA template, consisting of a 17-mer DNA primer annealed to M13 positive strand, resulting in the blocking of DNA synthesis by the Klenow fragment of Pol I. Auto-poly-ADP-ribosylated ADP-ribose transferase, which is the probable physiological state of this protein in intact cells, does not bind to primer-template DNA and does not block DNA synthesis by the Klenow fragment. On the basis of this in vitro model it is proposed that molecules which inhibit or inactivate ADP-ribose transferase in intact cells can induce significant alteration in DNA structure and replication.

1. INTRODUCTION

The major cell biological action of inhibitory ligands of the poly (ADP-ribose) polymerase activity of ADPRT (ADP-ribose transferase, E.C.2.4.4.30.) consists of a cell cycle dependent prevention of carcinogen-induced malignant transformation of human fibroblasts (1), conferring also carcinogen resistance (2), inhibition of malignant transformation in hamster embryo and mouse C3H10T1/2 cell cultures (3), deletion of transfected oncogenes from NIH 3T3 cells (4), suppression of the mitogenic stimulation of tumor promoters (5), inhibition of illegitimate DNA recombinations (6) and integration (7), induction of sister chromatid exchange (8) and the loss of certain amplified oncogenes (9,10). The molecular structures of inhibitory ADPRT ligands vary from benzamide (1), substituted benzamides (3,5,7,9, 10), 3-aminonaphthylhydrazide (6), isoquinoline, and quercetin, to coumarin (1,2-benzopyrone) (2). We have demonstrated that 1,2-benzopyrone that binds to ADPRT at the DNA-recognizing domain inhibits both in cell cultures and in vivo the corticosteroid activated malignant cell transformation of a rat cell line containing an oncogene construct (11). The observed apparently pleiotropic action of inhibitory ADPRT ligands is not instantaneous and requires at least one to three cell cycles (1,2,11), indicating either a slow progression of cellular responses or possibly a biochemical transformation of certain ADPRT ligands to active molecular species, or both. It is known

that 1,2-benzopyrone in vivo is readily hydroxylated in the 3 and 7 positions (12) and we found that the 7-hydroxy-1,2-benzopyrone does not inhibit ADPRT (unpublished results) which probably explains the transient nature of the antineoplastic effect of 1,2-benzopyrone in vivo (11). However substitution of an amino group in position 6 (13) produced 6-amino-1,2-benzopyrone (6-ABP) whose ADPRT inhibitory action and antineoplastic effect in cell cultures was not transient (14). We have therefore further investigated the biochemical fate of 6-ABP and identified a novel oxidative metabolite, 6-nitroso-1,2-benzopyrone (6-NOBP), formed by mixed function oxidases of liver microsomes in vitro. The 6-NOBP molecule still inhibited ADPR-polymerase activity but also inactivated ADPRT by destabilization of one zinc ion from one of the two zinc fingers of this protein.

The following report deals with this mode of action of 6-NOBP in vitro, while possibly in vivo consequences of this reaction are subject to further investigation.

2. Experimental

6-Nitro-1,2-benzopyrone (6-NO₂BP) was purchased from Pfaltz & Bauer (Waterbury, CT), 2-mercaptoethanol, NAD⁺, NADPH, nucleotides, sodium tungstate, hydrogen peroxide, Tris and MES buffers from Sigma (St. Louis, MO), Centricon 30 from Amicon (Beverly, MA), and HPLC-grade solvents and all other chemicals (reagent grade) from Fisher (Santa Clara, CA). [³²P]NAD⁺ (250 Ci/mmol) and [³²P]dCTP (650 Ci/mmol) were obtained from ICN radio-

chemicals (Irvine, CA) and $^{65}\text{ZnCl}_2$ (8.24 mCi/mg) from Dupont/NEN (Wilmington, DE). M13mp18 single-stranded circular DNA, M13 sequencing 17-mer primer GTTTTCCCAGTCACGAC and the Klenow fragment of DNA polymerase I were from New England Biolabs (Beverly, MA).

6-ABP was prepared from 6-NO₂BP (13) and labeled in the 5-position with ^3H using methodology previously reported (11). The specific activity of tritiated 6-ABP was 19 Ci/mmol. 6-NOBP was synthesized by the oxidation of 6-ABP (4 g/40 ml H₂O) at 22 °C by 30% H₂O₂ (5 ml) with sodium tungstate (5.93 g, in 20 ml H₂O) for 1.5 h followed by extraction of the green product into ethyl acetate, washing with 0.1 N HCl, and evaporation of solvent. Recrystallization from warm ethanol gave 1.48 g (42%) of product with a λ_{max} at 750 nm, characteristic of monomeric aryl nitroso compounds (15). Mass spectrum: m/z (relative intensity): 175 (M⁺, 100), 161 (16.88), 145 (33.77), 133 (10.38), 117 (56.09), 89 (79.71), 63 (57.13). High resolution data for the M⁺ peak: calculated for C₉H₅NO₃: 175.0268; found: 175.0271 (deviation = 1.1 ppm). ^1H -NMR (CDCl₃, 300 MHz) δ (ppm) from TMS: doublet (6.572 and 6.604) H-4 split by H-3; doublet (7.472 and 7.501) H-8 split by H-7; doublet of doublets (7.860/7.866 and 7.889/7.896) H-7 split by H-8 and finely split by H-5; doublet (7.910 and 7.942) H-3 split by H-4; doublet (8.308 and 8.315) H-5 finely split by H-7. UV/Vis spectrum in ethanol, λ_{max} (ϵ): 750 nm (46.0), 316 nm (8.96×10^3), 274 nm (2.24×10^4). M.p.: compound polymerizes above 160 °C, blackens and melts in the range of 325-340 °C.

Electrophoretically homogeneous ADPRT was isolated from calf thymus by published methods (16,17), and for loading with $^{65}\text{Zn}^{2+}$

the enzyme (770 μ g) was incubated in 500 μ l of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM 2-mercaptoethanol with 45 μ Ci $^{65}\text{Zn}^{2+}$ for 120 h at 6 $^{\circ}\text{C}$, at which time the $^{65}\text{Zn}^{2+}$ content of the protein was in equilibrium with the $^{65}\text{Zn}^{2+}$ in the exchange buffer (see Fig. 3). The time course of Zn^{2+} loading of ADPRT was determined by simultaneously monitoring the specific radioactivity of ADPRT-bound $^{65}\text{Zn}^{2+}$ chemically (18) and radiochemically. Then ADPRT solution was concentrated to a protein concentration of 3 mg/ml in Centricon 30 and stored at -20°C in the presence of 20% glycerol. Ejection of $^{65}\text{Zn}^{2+}$ from $^{65}\text{Zn}^{2+}$ -loaded ADPRT by 6-NOBP was assayed in 50 mM MES buffer (pH 6.0) containing 150 mM NaCl, 5 mM 2-mercaptoethanol and varying concentrations of 6-NOBP. Enzyme-bound $^{65}\text{Zn}^{2+}$ was measured by a filter binding assay (19). Enzymatic assays for ADPRT activity (13,16) and for DNA synthesis on a primer annealed to M13 positive strand DNA were carried out by published methods (20).

The biological oxidation of 6-ABP was measured by incubating for 30 min (37 $^{\circ}\text{C}$) 10 ml of a reaction mixture composed of 0.1 mM [^3H]6-ABP (26.7 mCi/mmol), 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 0.25 mM KCl, 1 mM NADPH and 1 mg/ml of rat liver microsomes (21) which were isolated from Fisher male rats pretreated with Aroclor 1254 as reported (22). The reaction was terminated by addition of an equal volume of cold acetone, and the mixture extracted with four 10 ml volumes of ethyl acetate, which combined were dehydrated over anhydrous sodium sulfate and evaporated to dryness by a stream of nitrogen. An aliquot of the residue

dissolved in acetonitrile/water (1:1 v/v) was analyzed by HPLC with an elution system employing three solvents (A = 0.05 M potassium phosphate, pH 6.0; B = same as A + 30% methanol; C = 50% acetonitrile, 50% water) at a flow rate of 1.2 ml/min using a reversed-phase ODS column by a published method (23). Analytical standards of 6-ABP, 6-NO₂BP and 6-NOBP eluted at 22.2 min, 33.5 min and 39.2 min, respectively. The identity of metabolites was established by their UV spectra and retention times which were identical with the authentic standards.

3. RESULTS

When [³H]6-ABP was oxidized by microsomes for 30 min, HPLC analysis identified the main oxidation product (7% of 6-ABP) as 6-NOBP (Fig. 1), with 2% of 6-ABP oxidized to 6-NO₂BP formed simultaneously (not shown). Among the oxidation products of 6-ABP only 6-NOBP was an ADPRT inhibitor, and in an ADPRT assay (13) with nM NAD as substrate and a synthetic octamer as coenzyme, 6-NOBP inhibited ADPRT with a k_i of 40 μ M, as compared with k_i for 6-ABP of 28 μ M, indicating that binding to ADPRT still occurs. In addition there was also an unexpected time-dependent inactivation of the poly (ADP-ribose) synthesizing activity of ADPRT by 6-NOBP. Incubation of ADPRT with increasing concentrations of 6-NOBP (Fig. 2) for 2 h in the presence of 5 mM 2-mercaptoethanol at 22°C and pH 6.0 resulted in a precipitous decrease of enzymatic activity coinciding with an ejection of ⁶⁵Zn²⁺ from the ⁶⁵Zn²⁺-loaded enzyme. The presence of 2-mercaptoethanol protected -SH groups of ADPRT against oxidation by the

nitroso compound and favored a selective attack on the zinc finger complexes of ADPRT. Coincidental with an almost total loss of polymerase activity nearly half of the Zn^{2+} content of ADPRT was ejected by the incubation with 6-NOBP, an effect which was not altered by added coenzymic DNA (cf. 16). Either 0.5 mM ZnCl_2 or CdCl_2 protected against inactivation by 6-NOBP (top curve, inset Fig. 2), but no protection was exerted by Fe^{2+} , Co^{2+} , or Mg^{2+} (not shown). The 6-NOBP-induced zinc loss was reversible in vitro, but reactivation, within the same time frame as Zn^{2+} ejection, required simultaneous incubation with added Zn^{2+} , dithiothreitol and DNA (results not shown). A correct interpretation of the 6-NOBP-induced zinc ejection from preloaded ADPRT critically depends on equilibration between externally added $^{65}\text{Zn}^{2+}$ and both enzyme-bound Zn^{2+} . As shown in Fig. 3, this was indeed attained since both Zn^{2+} of the enzyme protein exchanged with $^{65}\text{Zn}^{2+}$, albeit with two apparently distinct rates, as determined by assaying the specific radioactivity of ADPRT-bound Zn^{2+} as a function of time of incubation with $^{65}\text{Zn}^{2+}$.

A quantitative measure of the binding of ADPRT to DNA termini was obtained by determining the inhibition of DNA synthesis on M13 ssDNA annealed primer (20) as illustrated in Fig. 4. Untreated ADPRT inhibited DNA synthesis as a function of ADPRT concentration and at 0.5 μM ADPRT nearly complete inhibition occurred ($I_{50} = 0.17 \mu\text{M}$). Removal of one Zn^{2+} ion per molecule of ADPRT reduced the inhibitory effect of ADPRT ($I_{50} = 0.5 \mu\text{M}$) but did not abolish it. ADPRT has no direct effect on the Klenow enzyme and in this system inhibition is solely due to the binding

of ADPRT to 3' ends of DNA primers as also tested with coenzymic DNA (not shown). It is of interest that ADPRT concentration in nuclei is 1 μ M (28) therefore these in vitro results have biological relevance. Based on the similarity to results with site-directed mutation of zinc finger FI (29) 6-NOBP appears to destabilize Zn^{2+} preferentially from FI. A direct testing of this conclusion will require a significant quantity of ADPRT mutated in FI, which has thus far been assayed in cell extracts only (29).

Since ADPRT in differentiated cells exists mainly in the auto-poly-ADP-ribosylated form (30), the effect of the in vitro auto-poly-ADP-ribosylated enzyme protein on DNA synthesis at the primer template site was also determined. As shown in Fig. 5 (upper curve) the auto-modified enzyme completely lost its inhibitory effect at the primer-template site. In separate experiments it was found that as few as 35 ADP-ribose units per molecule of ADPRT were sufficient to abrogate the inhibitory action of ADPRT on DNA synthesis (not shown).

The 29 kDa terminal polypeptide of ADPRT (31) which contains both zinc fingers (32,33) was slightly less than 50% as effective an inhibitor, when present in equimolar concentration with ADPRT (Fig. 5, middle curve), whereas the 36 kDa and 56 kDa polypeptide components of ADPRT (31) had no detectable inhibitory action at equimolar concentrations with the intact enzyme molecule (results not shown).

4. Discussion

The oxidation of 6-ABP to 6-NOBP by microsomal mixed function oxidases (Fig. 1) may not be confined to one system since other O_2^- generating enzymatic reactions (e.g. in granulocytes) predictably can also perform this oxidation, a problem which is currently studied. In intact cells the steady state concentration of enzymatically generated 6-NOBP should be very low because of its facile reduction to 6-ABP by GSH and other reducing systems, except at membrane-associated sites of its formation, presumably at the nuclear membrane where ADPRT also resides (24,25), a locus which is contiguous with the endoplasmic reticulum (26,27) containing oxidases. It is also possible that the cellular pharmacologic action of 6-ABP is probably a composite effect of ADPRT inhibition by 6-ABP itself to which a progressive inactivation of ADPRT by locally generated 6-NOBP is superimposed. The exact nature of this combined effect in cellular systems is not known. On the other hand, on the basis of present results the consequences of ADPRT inhibition and inactivation are predictable. Since the turnover of poly (ADP-ribose) in intact tissues is relatively slow ($t_{1/2}$ is between 2.6 and 4 h in vivo, cf. 39) a progressive removal of the DNA-repelling (40) poly (ADP-ribose) from ADPRT by catabolic enzymes will take place following the inhibition and inactivation of poly (ADP-ribose) synthesis by ADPRT ligands. Thus a time-dependent emergence of de-ADP-ribosylated ADPRT occurs, which is then available for binding to specific DNA structures and complex cellular responses may ensue (1-11).

The most probable mechanism of the destabilization of Zn^{2+} from ADPRT by 6-NOBP is the oxidation of cysteine ligands (29) in the zinc finger peptide, forming disulfide and 6-hydroxylamino-1,2-benzopyrone, similar to the reactivity of other nitroso compounds (38). As expected, other arylnitroso compounds can also destabilize Zn^{2+} in ADPRT, but among the molecules so far tested (nitrosobenzene, 4-nitrosophenol, 1-nitroso-2-naphthol-3,6-disulfonic acid) 6-NOBP proved to be the most effective, probably because of its binding affinity to ADPRT.

The facile exchange of $^{65}\text{Zn}^{2+}$ with the two enzyme-bound Zn^{2+} ions of ADPRT, as illustrated in Fig. 3, is not a universal property of all zinc finger proteins, which frequently require pretreatment with organomercurials (34,35) for Zn^{2+} release. However in both cases cited (34,35) only one of the zinc fingers was destabilized by p-hydroxymercuriphenylsulfonate, similar to our observations with 6-NOBP in ADPRT. In our experience p-hydroxymercuribenzoate removed both Zn^{2+} ions of ADPRT and denatured ADPRT, in contrast to 6-NOBP which reversibly destabilized one Zn^{2+} ion (Fig. 2). The apparently biphasic Zn^{2+} exchange in ADPRT (Fig. 3) may suggest dissimilarity between the two zinc fingers. Dissimilarity of zinc fingers within the mammalian glucocorticoid receptor was earlier indicated by their differing NMR relaxation times (36). The need for individual assessment of zinc binding polypeptide structures has been recently emphasized (37). If the in vitro detectable relative ease of Zn^{2+} exchange exists in cellular systems, ADPRT may be a biological Zn^{2+} sensor, a question which requires further studies.

The 29 kDa polypeptide of ADPRT by itself has similar but weaker template inhibitory action than ADPRT (Fig. 5), suggesting that the second DNA binding polypeptide of ADPRT (36 kDa, cf. 31), which by itself is not inhibitory, in the intact ADPRT molecule apparently reinforces the binding of the adjacent zinc finger domain to DNA termini. The significance of the second (36 kDa) DNA binding domain of ADPRT (31) has been emphasized by the recognition of a helix-turn-helix motif in the cDNA of ADPRT, coding for the 36 kDa polypeptide (41).

Acknowledgments

This work was supported by grants from the US Air Force Office of Scientific Research (AFOSR-89-0221) and the Aaron Diamond Foundation (New York University School of Medicine) and by Octamer Inc. (Tiburon, CA, USA). We thank Dr. Ursula Simonis of the Department of Chemistry and Biochemistry, San Francisco State University, for measuring ^1H -NMR spectra. The helpful comments of Dr. H.G. Williams-Ashman (University of Chicago) are acknowledged with thanks.

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Figure Legends

Figure 1. Identification by HPLC of [^3H]6-NOBP as the main oxidation product of [^3H]6-ABP catalyzed by rat liver microsomes. The analytical standard 6-NOBP (solid line) was monitored by UV absorbance (left ordinate) and the enzymatic product (dashed line) by [^3H]-radioactivity (right ordinate) with the chromatographic retention time shown on the abscissa. The UV spectra of the eluate peak and 6-NOBP standard, as measured by the diode-array detector, were identical (not shown). Details of the incubation system and HPLC procedure are given in Experimental.

Figure 2. The effects of incubation of ADPRT with 6-NOBP on the enzymatic activity and $^{65}\text{Zn}^{2+}$ content of the ADPRT protein. The ordinate shows the percentage changes in enzymatic activity (100% is 231 pmol ADPR/ μg protein/min) and in $^{65}\text{Zn}^{2+}$ content (see Fig. 3) following 2 h incubation of $^{65}\text{Zn}^{2+}$ -loaded ADPRT (17) with varying concentration of 6-ABP (abscissa) in a system composed of 50 mM MES-buffer (pH 6.0), 100 mM NaCl, 0.5 mM EDTA and 5.0 mM 2-mercaptoethanol and varying concentrations of 6-NOBP in a final volume of 10 μl at 25°C. A 1 μl aliquot was used for ADPRT assay (see Experimental) and the remaining material for the radiochemical test for $^{65}\text{Zn}^{2+}$ content of ADPRT (19). Closed circles indicate $^{65}\text{Zn}^{2+}$ content[^], open circles ADPRT activity. Inset shows the time course of the effect of 0.5 mM 6-NOBP on ADPRT activity (open circles) and $^{65}\text{Zn}^{2+}$ content (closed circles), while the open triangles indicate the protective effect

of 0.5 mM ZnCl_2 on ADPRT activity in the presence of 0.5 mM 6-ABP. The pH 6.0 was chosen for 6-NOBP induced $^{65}\text{Zn}^{2+}$ ejection because its rate was maximal at that value but the induced $^{65}\text{Zn}^{2+}$ efflux from ADPRT occurs also at pH 7.0, with a rate reduced to 30%. All assays were done in triplicates, which agreed within 10%.

Figure 3. Exchange of externally added $^{65}\text{Zn}^{2+}$ with ADPRT-bound Zn^{2+} as a function of time of incubation. ADPRT (700 μg) was incubated at 6°C in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol to which 45 μCi of $^{65}\text{Zn}^{2+}$ (1.12 mCi/ μmol) was added. The $^{65}\text{Zn}^{2+}$ and Zn^{2+} content of ADPRT were determined using published radiochemical (19) and chemical (18) methods. Assays were done in duplicates with $\pm 10\%$ variation.

Figure 4. Inhibition of DNA synthesis by varying concentrations of native ADPRT (closed circles) and 6-NOBP-treated Zn^{2+} -deficient ADPRT protein (open circles). The ordinate shows DNA polymerase activity as % of controls, assayed as [^{32}P]dCMP incorporation (30 min) by the Klenow fragment of DNA polymerase I with M13 ssDNA annealed template primer as reported (20). The 100% value for Klenow polymerization is 112 pmol of nucleotides incorporated per 30 min in 50 μl reaction mix (25 °C). Duplicates agreed within 10%.

Figure 5. The effects of ADPRT (open circles), auto-poly-ADP-ribosylated ADPRT (closed circles), and the isolated 29 kDa polypeptide (open triangles) on DNA synthesis on the M13 ssDNA annealed primer (20) by the Klenow enzyme. ADPRT was auto-poly-ADP-ribosylated by preincubation with 0.1 mM NAD^+ at pH 8.0 for 10 min at 25 °C in the presence of all components, except the Klenow enzyme which was added after 10 min to initiate DNA synthesis, which lasted for 30 min. Triplicate assays agreed within 10%.

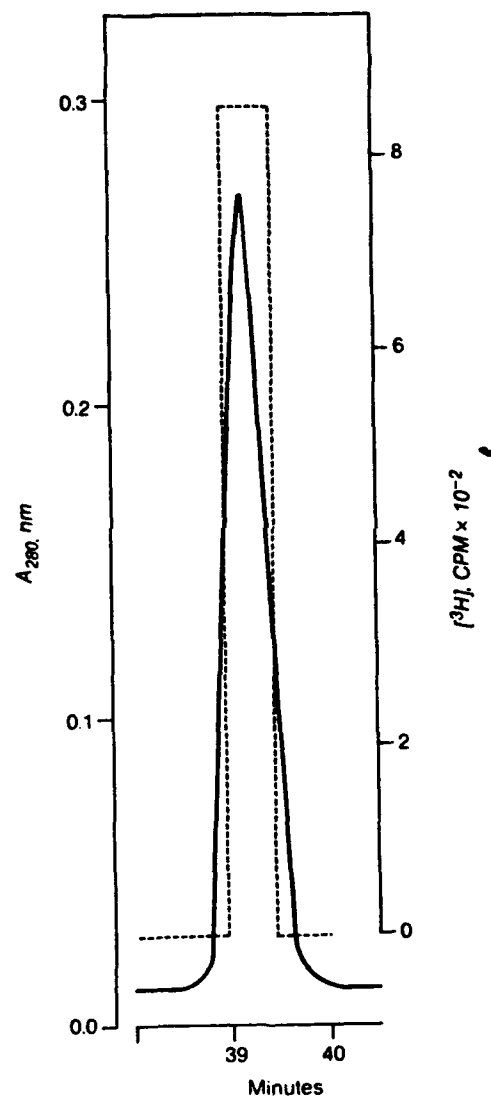


Fig. 1

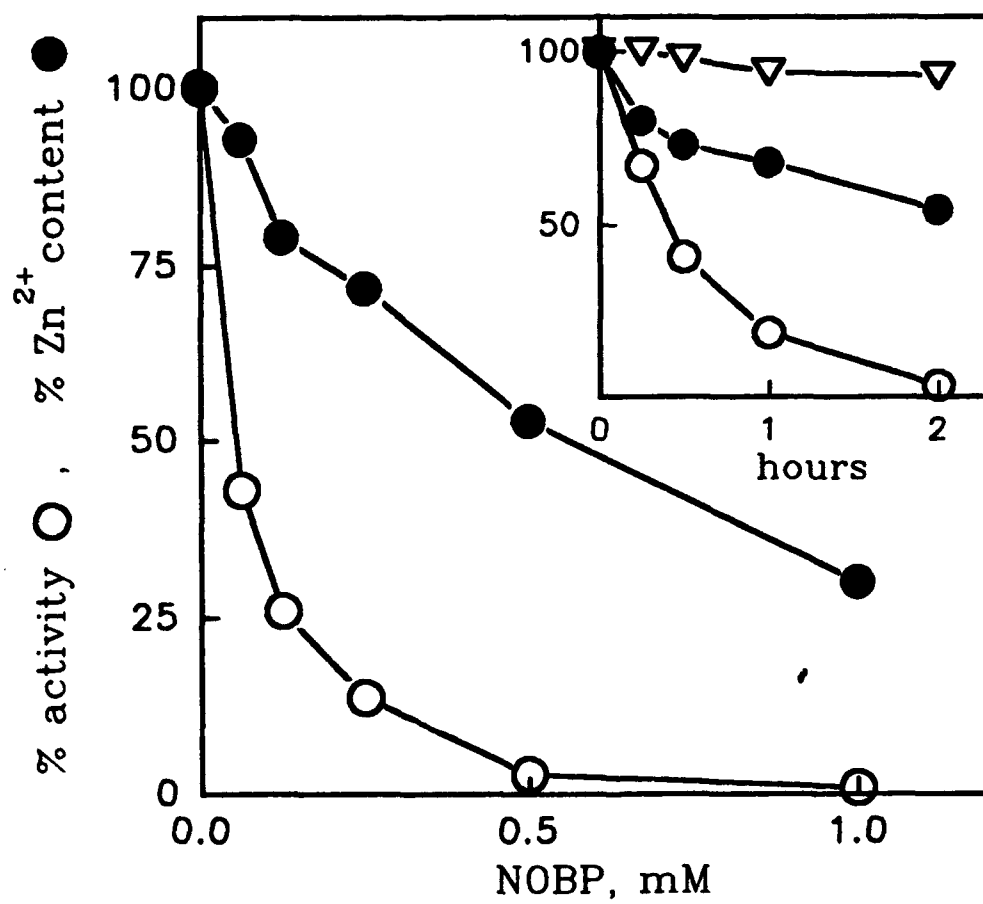


Fig. 2

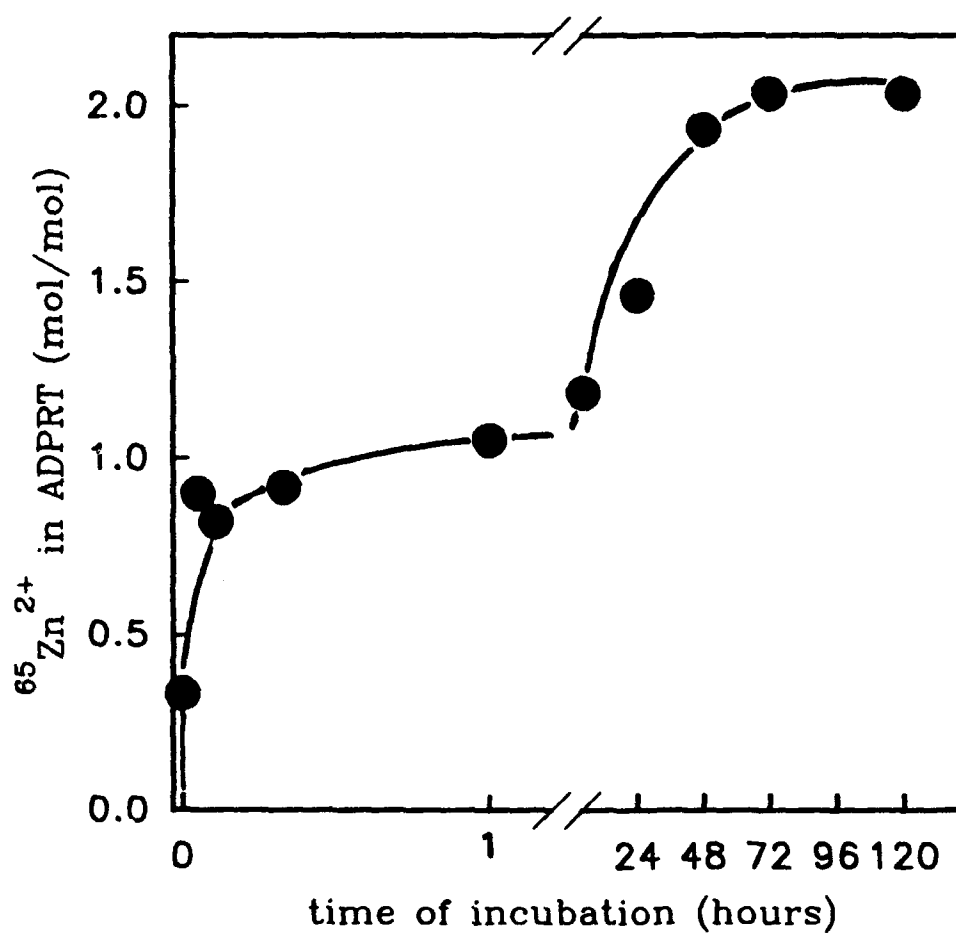


Fig. 3

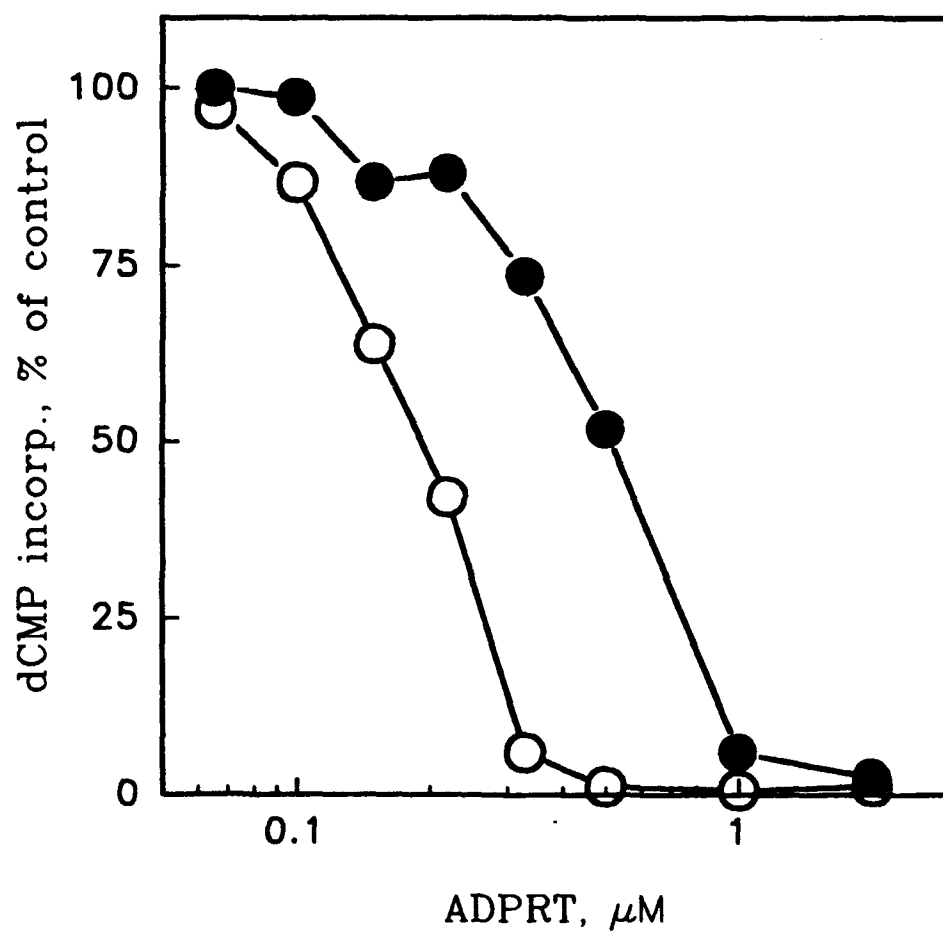


Fig. 4

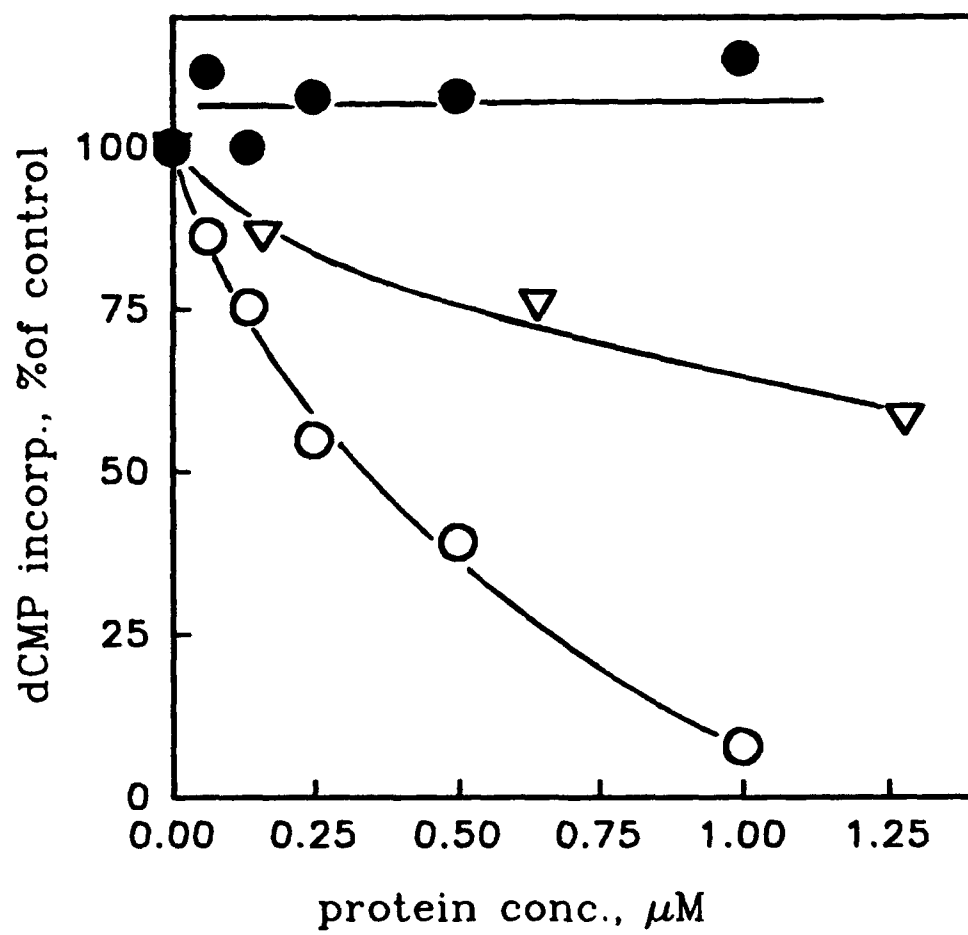


Fig. 5

III

Inhibitory Binding of Adenosine Diphosphoribosyl Transferase
to the DNA Primer Site of Reverse Transcriptase Templates.

Purified adenosine diphosphoribose transferase protein binds to RNA-DNA hybrid templates of reverse transcriptase at the DNA primer site and inhibits RT activity of HIV and MMu RTs. This action is prevented by auto-poly-ADP-ribosylation of the transferase but is reinduced by inhibitory ligands of the enzyme.

Adenosine diphosphoribosyl transferase (ADPRT, E.C.2.4.2.30) is a highly abundant non-histone nuclear protein of higher eukaryotes and there is convincing evidence that the poly(ADP-ribose) synthesizing function of this protein represents only a few percent of its molecular activity in intact cells (1,2). This is in agreement with the magnitude of the DNA-independent rates of oligo(ADP-ribose) synthesis (3), which can be readily determined even in the 56 kDa polypeptide fragment of ADPRT (4) that has no DNA recognition sites (5). Consequently the relatively slow auto-poly-ADP-ribosylation of ADPRT and slow turnover of the polymer in intact cells (6) serves most probably a self-regulatory function by providing the DNA-repelling $(ADPR)_n$ that is covalently bound to ADPRT (7). Considerable evidence exists demonstrating the binding of ADPRT to DNA termini as well as to internal DNA regions and cruciform DNA (10,11), and significant topological changes result from the binding of ADPRT to circular DNA, a process that is also regulated by both NAD and enzyme inhibitory ligands (12). Recently, it has been shown that ADPRT has a regulatory effect on the synthesis of SV40 DNA (28), and it

Abbreviations: ADPRT, Adenosine diphosphoribosyl transferase; RT, reverse transcriptase; TE, Tris-EDTA buffer; NA, nucleic acid; GSH, reduced glutathione; DTT, dithiothreitol; $(ADPR)_n$, polyadenosine diphosphoribose; MMu RT, Moloney murine leucosis virus RT.

has also been demonstrated that in vitro DNA synthesis by the Klenow fragment is inhibited by ADPRT (8), and this inhibition modulated by auto-ADP-ribosylation of ADPRT or by removal of Zn^{2+} from one of its two zinc fingers (9).

The present report, as an extension of previous studies, deals with the binding of ADPRT to RNA-DNA hybrids, in particular to primer sites of reverse transcriptase templates.

Materials and Methods

MMu RT, p(rA).(dT)₁₂₋₁₈ and p(rC).(dG)₁₂₋₁₈ were obtained from Pharmacia (Piscataway, NJ), and polyA (average length of 300 b), histones (Cat. #7755), oligo(dT)₁₂₋₁₈, dGTP, and TTP from Sigma (St. Louis, MO). Tracer nucleotides [α -³²P]TTP, [α -³²P]dGTP (each 650 Ci/mmol) and [³²P]NAD (250 Ci/mmol) were purchased from ICN (Irvine, CA). ADPRT was prepared from calf thymus by a published method (14). HIV reverse transcriptase (recombinant, clone BH-10) was a gift from the AIDS Research and Reference Reagent Program of AIDS, NIAID, NIH (Rockville, MD). Octameric dsDNA (GCATGCAT) was prepared by annealing synthetic complementing strands (15). Coenzymic DNA (CoDNA) consisted of 200-1000 bp dsDNA, a side product of the isolation of ADPRT (13), with an average of 500 bp (325 kDa) size. Nitrocellulose membranes (type HA, 2.4 cm diameter, 0.45 μ m) were from Millipore (Bedford, MA) and filter binding assays were performed as published (12). The labeled hybrid polynucleotide p(rA).p([³²P]dT) was prepared by annealing 66 μ g of poly(rA) with 0.8 μ g oligo(dT)₁₂₋₁₈ in 40 μ l of TE buffer at room temperature for 30 min, then 200 μ l of 5xHIV buffer (250 mM Tris-HCl, 300 mM KCl, 35 mM MgCl₂, pH 8.2) and 70 μ Ci of [α -³²P]TTP and 650 μ l of sterile water were added. Finally 0.5 μ g of HIV-RT in 100 μ l of RT diluter (50 mM phosphate, pH 7.2, 50 mM KCl, 50% glycerol and 0.2% Triton X-100) was admixed and the system incubated at 25°C for 40 min, followed by extraction with phenol/chloroform, 1 : 1(v/v) and with chloroform. The nucleic acid was precipitated with 2 volumes of 100% ethanol overnight at -20°C, centrifuged and the precipitate dissolved in 40 μ l of TE buffer. Spectrophotometric assay for nucleic acid concentration was based on 1 A₂₆₀ = 40 μ g nucleic acid, and the specific activity was 28,500 cpm/ μ g. Gel retardation was assayed in both 1.2% agarose and 4% acrylamide gels as described (16). Assays for ADPRT activity were done in aliquots (50 μ l) containing 16.5 pmol of ADPRT in 1xHIV buffer supplemented with 8 mM DTT, 2 mM GSH, and 0.1 mM [³²P]NAD (0.1 μ Ci). After incubation at 25°C for 4 min, the reaction was stopped by 2 ml of 20% trichloroacetic acid and the precipitate collected by filtration and radioactivity counted by liquid scintillation spectrometry (13). The ADP-ribosylated ADPRT was made in 6 ml of reaction mix containing 25 mM Tris.HCl (pH7.7), 1 mM EDTA, 0.25 mM NAD, 0.2% Tween 20, 240 μ g of ADPRT and 0.1 μ g of octameric dsDNA. After incubation for 2 hours at 25°C, the mix was concentrated in

Centricon 30 (Amicon, Beverly, MA) to 3 mg/ml and the medium changed to 25 mM Tris.HCl (pH 7.7), 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.2% Tween 20 with the concomitant removal of the octameric DNA and NAD. HIV RT activity was assayed in 50 μ l of 1xHIV buffer supplemented with 8 mM DTT, 2 mM GSH, 50 μ M [α - 32 P]TTP or [α - 32]dGTP and 2 μ g of p(rA).(dT)₁₂₋₁₈ or p(rC).(dG)₁₂₋₁₈; 0.03 μ g of HIV RT started polymerization at 25°C, which was stopped after 10 min with cold TCA, precipitate collected on filters, and radioactivity counted as for ADPRT assays above. In experiments (Fig. 3B, 4B), where the concentration of (dT)₁₂₋₁₈ primer was varied, the poly(rA) was kept constant (3.3 μ g/tube) and annealing was allowed for 30 min at ambient temperature in TE buffer prior to addition of other components of the RT assay. With MMu RT, the reaction was done at 37°C. RNase H activity was tested as published (17) and partially zinc-depleted ADPRT was prepared as reported (9).

Results

Binding constants of ADPRT and HIV RT to the poly(rA).(dT)₁₂₋₁₈ hybrid were determined by a filter binding technique (12) in a system containing 1.5 nM ADPRT or HIV RT and varying concentrations of the hybrid between 0.075 and 3 μ g/ml. Scatchard binding analysis revealed high affinity K_d values, namely 0.26 ± 0.1 nM for ADPRT and 0.31 ± 0.1 nM for HIV RT, as determined in 5 parallel tests.

Binding of ADPRT to the hybrid nucleic acid was also demonstrated with the gel retardation assay (Fig. 1.) The first 5 lines show the retardation of the labeled hybrid by increasing concentrations of ADPRT, whereas preincubation of ADPRT with 1 mM NAD - its substrate - completely abolished retardation (lanes 6-10). When the enzymatic activity of ADPRT was inhibited by either benzamide (18) or 5-iodo-6-amino-1,2-benzopyrone (19) ADPRT was bound to the hybrid as shown by unimpaired retardation (lanes 11-20). It was determined in separate tests that benzamide at 0.4 mM or 5-I-1,2-benzopyrone at 0.5 mM had no direct effect on isolated RT enzymes (not shown). HIV RT also retarded the migration of the hybrid (not shown). Since binding of ADPRT to DNA termini generally increases the polymerase activity of ADPRT, we compared the coenzymatic function of the hybrid with known activators of

ADPRT, coenzymic DNA (14) and a synthetic octameric DNA duplex (15). Results are illustrated in Fig. 2 wherein it is evident that despite the strong binding of the hybrid to ADPRT, only very small coenzymatic activation of the poly(ADP-ribose) polymerase function of ADPRT is conferred by the hybrid.

The suppressive effect of ADPRT on HIV RT activity is illustrated in Fig. 3 (A and B). Two different templates were used and results show a stronger suppression with p(rA).(dT)₁₂₋₁₈ than with p(rC).(dG)₁₂₋₁₈; I₅₀ of ADPRT is 0.3 μ M for the former and slightly above 1 μ M for the latter template. The preference of binding of ADPRT to p(rA).(dT)₁₂₋₁₈ agrees with results obtained with various dsDNAs (10). The apparent kinetics of inhibition of HIV RT by ADPRT simulates competition (Fig. 3B), presumably a competition between RT and ADPRT for the primer site of the hybrid. It is of interest that histones reinforce the inhibitory action of ADPRT (top curve of 3B; compare with second curve from top) whereas histones themselves have no direct inhibitory action on HIV RT (compare lower two curves). Poly-ADP-ribosylated ADPRT has no inhibitory action on HIV RT (Fig. 4A), and partial depletion of Zn²⁺ from ADPRT, by pretreatment with 6-nitroso-1,2-benzopyrone (9), diminishes but does not abolish the inhibitory binding of ADPRT to HIV RT (Fig. 4B). These results are comparable to the influence of partial zinc depletion of ADPRT on its inhibitory effect on the DNA polymerase activity of the Klenow enzyme (9). The effect of ADPRT on MMu RT activity is summarized in Table 1. Just as with HIV RT, histones promote the inhibitory action of ADPRT without being by themselves inhibitory, and preference of p(rA).(dT)₁₂₋₁₈ to p(rC).(dG)₁₂₋₁₈ was also shown. Contrary to the DNA synthetic activity of HIV RT, its RNase H activity was unaffected by ADPRT (results not shown).

Discussion

The inhibitory effect of ADPRT on DNA synthesis at the primer site of RT template illustrates a novel binding of ADPRT to RNA-

DNA hybrids. Despite the strong binding, the hybrid nucleic acid has only minimal coenzymic efficacy on ADPRT. This observation adds to already accumulating evidence that colligative properties per se of ADPRT probably play a prominent role in physiologically functioning cells and that enzymatic (ADPR)_n formation is a self-regulatory activity of ADPRT. The concentrations of ADPRT at which we observe significant inhibition of RT are well within the actual ADPRT concentration range existing in nuclei, which is 1 μ M (20). Therefore we conclude that a drug-induced binding of ADPRT to RT templates within the cell may have pharmacological relevance. It is generally thought that ADPRT is a predominantly nuclear enzyme (21) whereas retroviral RT is mainly operative in the cytosol (22). However evidence exists that the nuclear localization of ADPRT is not exclusive, inasmuch as cytoplasmic ADPRT species (23-26) were identified as well as (ADPR)_n glycohydrolase (27) in isolated ribonucleoprotein particles. Therefore a cellular antiretroviral action of ADPRT ligands, which may induce the binding of ADPRT to RT templates, cannot be excluded, an example of which is reported in an accompanying paper (19).

Acknowledgments

This research was supported by AFOSR 89-0231 and by funds of Octamer Research Foundation. The following reagent was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH: HIV reverse transcriptase from the Univ. of Alabama at Birmingham, Center for AIDS Res., Gene Expression Core Facility.

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Figure Legends

Figure 1. Gel retardation assay for the binding of ADPRT to p(rA).p([³²P]dT) hybrid polynucleotide. Gel: 1.2% agarose. NA: 35 ng (1000 cpm)/lane. Lanes 1-5 show the retardation of labeled hybrid NA by increasing amounts (0, 5, 10, 20, 40 ng) of ADPRT. (Note: due to a handling error, lane 4 contained 40 ng and lane 5 20 ng of ADPRT) Lanes 6-10 are with the same progression of ADPRT amounts but preincubated with 1 mM NAD for 10 min at ambient temp; lanes 11-15 are the same but with benzamide added (0.4 mM); while lanes 16-20 have 5-iodo-6-amino-1,2-benzopyrone (0.5mM) added.

Figure 2. Coenzymic activity of CoDNA, octameric deoxyribonucleotide duplex (octamer DNA) and p(rA).(dT)₁₂₋₁₈ on the rates of auto-poly-ADP-ribosylation of ADPRT. Assay for ADPRT activity described in Methods.

Figure 3. A: The inhibitory effect of ADPRT on HIV RT at varying concentrations of ADPRT with two types of RNA-DNA hybrids as templates. Hundred % activities were 53 pmol and 21 pmol of nucleotides incorporated for the A.T and C.dG templates respectively, and 3 µg histones were present in all assays. **B:** Lineweaver-Burk plot of HIV RT activity at varying concentrations of primer. Ordinate (1/V) is in units of pmol⁻¹ of TMP incorporated in 10 min. O, no additive; ▽, plus histones (3 µg); ●, plus ADPRT (0.67 µM); ▼, plus histones and ADPRT.

Figure 4. A: The effect of native ADPRT and of auto-ADP-ribosylated ADPRT on HIV RT activity. Conditions were the same as in Fig. 3A, auto-ADP-ribosylated ADPRT was made as described in Methods, 100% activity: 65 pmoles TMP incorporated. **B:** The effects on HIV RT activity of native ADPRT and ADPRT which is 50% zinc-depleted by treatment with 6-nitroso-1,2-benzopyrone (NOBP). Conditions were the same as in Fig. 3B.

Alternate layout for legend Fig 2.

Figure 2. Coenzymic activity of CoDNA, octameric deoxyribonucleotide duplex (octamer DNA) and p(rA).(dT)₁₂₋₁₈ on the rates of auto-poly-ADP-ribosylation of ADPRT. Assay for ADPRT activity described in Methods.

Table 1

The effect of ADPRT on the activity of MMu RT.

Exper. conditions	RT activity with	
	p(rC).(dG) ₁₂₋₁₈	p(rA).(dT) ₁₂₋₁₈
No ADPRT, No Histone	120 (100%)	56.8 (100%)
+ ADPRT, No Histone	81 (38% inhib.)	12.9 (87% inhib.)
No ADPRT, + Histone	109	58.2
+ ADPRT, + Histone	30 (75% inhib.)	6.7 (88% inhib.)

RT concentration was 0.02 μ g/test consisting of 50 mM Tris-HCl (pH 8.5 at 25°C), 8 mM MgCl₂, 10 mM DDT, 0.5 mM [α -³²P]TTP or [α -³²P]dGTP, 1 μ g of p(rA).(dT)₁₂₋₁₈ or p(rC).(dG)₁₂₋₁₈ in 50 μ l volume. ADPRT: 0.67 μ M; histones: 5 μ g/test, if present. Incubation was carried out at 37°C for 5 min and acid precipitable radioactive product assayed as described in Methods. Values in the table are given as pmoles of deoxyribonucleotides incorporated in 5 min at 37°C. Data are the averages of 3 parallel assays, not differing more than $\pm 10\%$ from the mean value.

Fig. 1

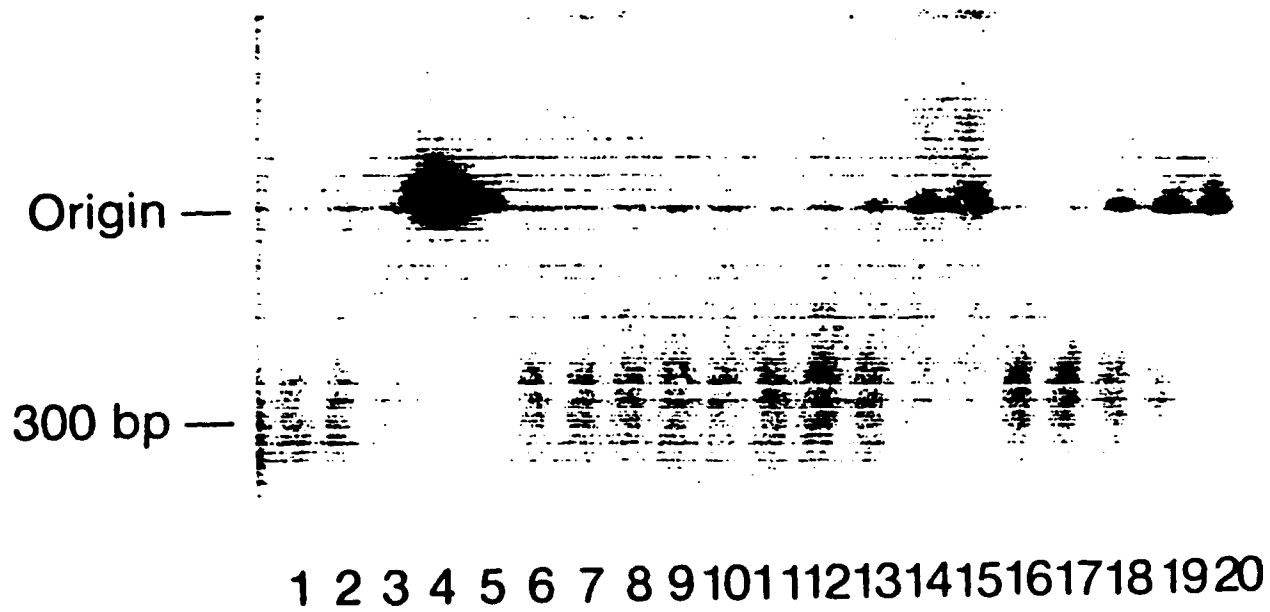


Fig 2 Plot of $\frac{\text{mol ADP-ribose incorp.}}{\text{mol ADPRT}}$ vs. nucleic acid, nM

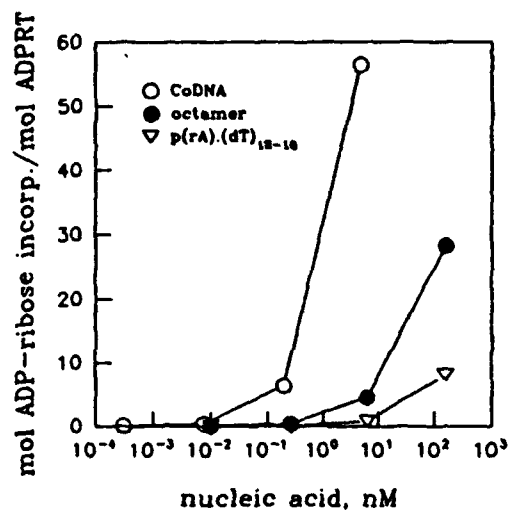
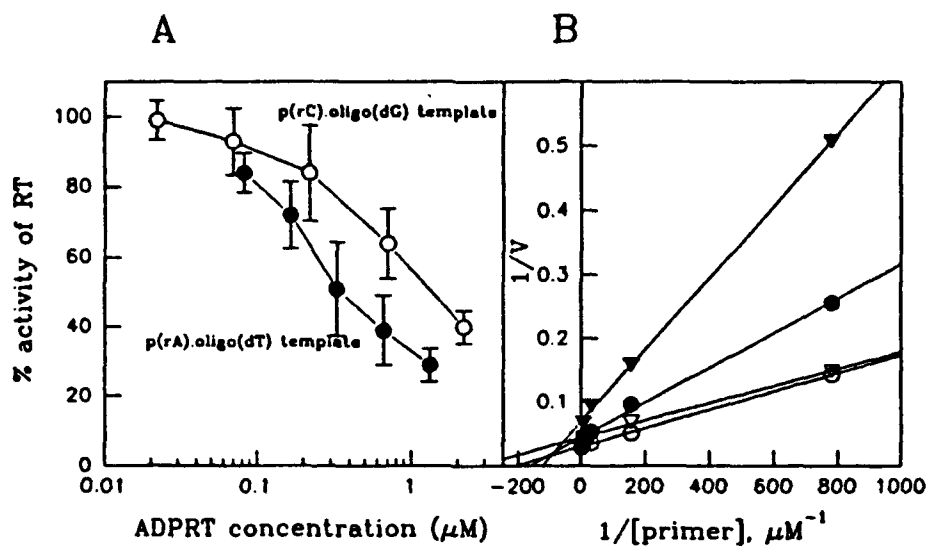
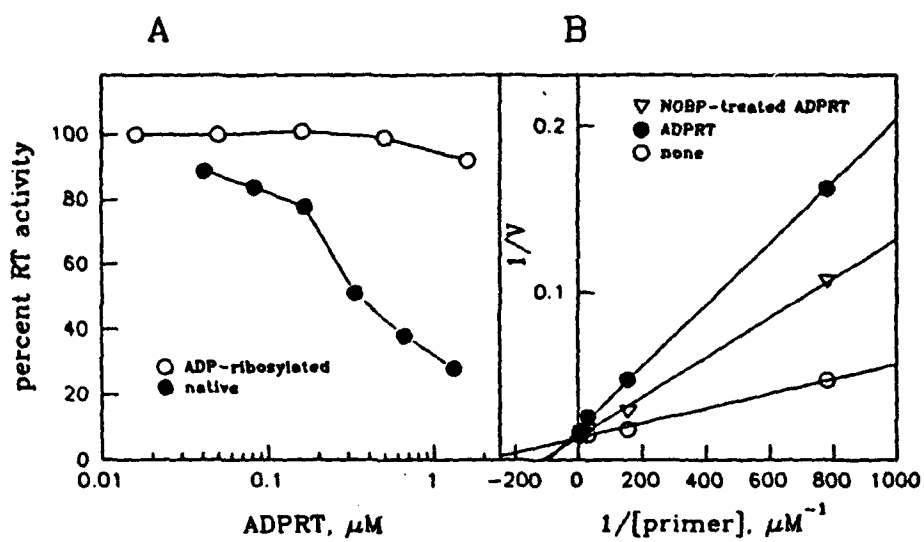


Fig 3

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IV

Inhibition of HIV-1 IIIb Replication in AA-2 and MT-2 Cells
in Culture by Two Ligands of Poly(ADP-Ribose) Polymerase: 6-
Amino-1,2-benzopyrone and 5-Iodo-6-amino-1,2-benzopyrone.

The effects of two adenosine diphosphoribose transferase (ADPRT) enzyme inhibitory ligands, 6-amino-1,2-benzopyrone and its 5-iodo-derivative, were determined in AA-2 and MT-2 cell cultures on the replication of HIV-1 IIb, assayed by an immunochemical test for the HIV protein p24, and syncytium formation, characteristic of HIV-infected cells. Intracellular concentrations of both drugs were sufficient to inhibit poly(ADP-ribose) polymerase activity within the intact cell. Both drugs inhibited HIV replication parallel to their inhibitory potency on ADPRT, but distinct differences were ascertained between the two cell lines. In AA-2 cells both p24 and syncytium formation were depressed simultaneously, whereas in MT-2 cells only syncytium formation was inhibited by the drugs, and the p24 production, which remained unchanged during viral growth, was unaffected. Both drugs only moderately depressed the growth rate of the AA-2 and MT-2 cells and there was no detectable cellular toxicity. Results suggest the feasibility of the development of a new line of ADPRT ligand anti-HIV drugs that fundamentally differ in their mode of action from currently used chemotherapeutics.

The major cellular effects of ligands of the nuclear protein adenosine diphosphoribose transferase (ADPRT, E.C.2.4.4.30) in intact human cells as well as in hamster embryo and mouse cells are a prevention of chemical and UV-induced carcinogenesis (1, 2), suppression of dexamethasone-induced malignant transformation in a rat cell line containing an oncogene construct (3), deletion

Abbreviations: ADPRT, adenosine diphosphoribose transferase; (ADPR)_n, polyadenosine diphosphoribose; HIV, human immunodeficiency virus; RT, reverse transcriptase; 6-ABP, 6-amino-1,2-benzopyrone; 5-IABP, 5-iodo-6-amino-1,2-benzopyrone; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine.

of transfected oncogenes from NIH 3T3 cells (4), inhibition of the integration of externally applied DNA into genomic DNA (5), induction of sister chromatid exchange (6), loss of certain amplified oncogenes (7,8) and inhibition of the development of fertilized eggs of *Mytilus edulis* (9). The poly(ADP-ribose) synthetase function of ADPRT in intact cells accounts only for 1% of the molecular activity of this enzyme (10,11) and the turnover of the polymer in intact cells is relatively slow (12); therefore additional activities of this protein were sought to explain its physiologic function. Colligative properties of ADPRT, particularly towards DNA (13-15), represent another molecular activity of ADPRT of potential significance. It has been shown that the unmodified ADPRT protein can bind to replicative DNA sites (16-18) and thus regulate replication. We have recently reported that in retinoic acid-induced differentiated cells ADPRT can exist as an auto-poly-ADP-ribosylated protein that cannot synthesize polymer but functions as a nuclear NAD glycohydrolase (19). Since it is known that $(ADPR)_n$ polymers repel DNA (20) it became apparent that a DNA binding function of poly-ADP-ribosylated ADPRT molecules can be induced when the synthesis of poly(ADP-ribose) is selectively blocked by inhibitors, which do not influence its catabolism (cf. 21). As we report in an accompanying paper, ADPRT also binds to RNA-DNA hybrid templates of HIV reverse transcriptase at the DNA replication origin (22), an effect that is prevented by auto-poly-ADP-ribosylation of the enzyme. It follows that certain ADPRT ligands by blocking poly(ADP-ribose) synthesis in the cell may indirectly induce the inhibition of viral replication through the binding of ADPRT to reverse transcriptase templates. The poly(ADP-ribose) synthesis is most probably activated by cuts in genomic DNA that occur during the integration process of retroviral DNA (34,35). Inhibition of poly (ADP-ribose) synthesis by diminishing the concentration of the covalently bound DNA-repellant $(ADPR)_n$ polymer may facilitate the binding of ADPRT to DNA or DNA-RNA templates.

The present report is concerned with the testing of this hypothetical mechanism in two cell lines, AA-2 and MT-2 cells (23), which are good supporters of HIV replication. Two inhibitors of ADPRT, 6-amino-1,2-benzopyrone (6-ABP) (24) and its 5-iodo-derivative (5-IABP), were found to be effective molecules against HIV replication and their respective potencies parallel their inhibitory action on the purified ADPRT or the activity of the enzyme in intact cells.

Materials and Methods

Virus. The HTLV-IIIB isolate of HIV-1 (25) consisted of clarified culture medium from a persistently infected human T cell line (MOLT-3, obtained from Dr. Max Essex, Harvard School of Public Health, Boston, MA). The cells were propagated in RPM-1640 medium, containing 10% heat-inactivated (56 °C, for 30 min) fetal bovine serum and antibiotics (100 U penicillin, 100 U streptomycin and 40 µg/ml gentomycin). Polybrene (Sigma, St. Louis, MO) was added to a final concentration of 0.15 µg/ml. The medium from a 4-day culture of infected MOLT-3 cells had an infectivity titer of 2 to 3 x 10⁵ per ml, as determined in AA-2 cells.

The adenosine diphosphoribose polymerase ligands, 6-amino-1,2-benzopyrone hydrochloride (6-ABP) was synthesized as reported (24) and the 5-iodo-6-amino-1,2-benzopyrone analog (5-IABP) was prepared by the iodination of 6-ABP hydrochloride with iodine monochloride in ethanol following a general method (26). The structure of the pure, crystalline product (m.p. 163-165 °C) was established by high resolution mass spectrometry and ¹H and ¹³C NMR spectrometry (30).

Cellular uptake of 6-ABP and 5-IABP was measured using [³H]-labeled 6-ABP prepared by a reported method (3) and [¹²⁵I]-labeled 5-IABP, which was prepared as follows. 6-ABP hydrochloride in ethanol was iodinated with [¹²⁵I]-labeled iodine monochloride (26) generated by equilibrating carrier-free sodium iodide in NaOH with iodine monochloride. The 5-[¹²⁵I]ABP was purified by preparative TLC (silica gel, ethyl acetate-hexane 1:1 v/v) and had a specific radioactivity of 2.8 mCi/mmol.

The AA-2 or MT-2 cells were incubated with the labeled ligands in Petri dishes (35 mm diameter) containing 3 ml culture medium, 1.5 x 10⁶ cells and labeled 6-ABP (3 mM) or 5-IABP (0.3 mM) for 18 hr to obtain equilibrium. Thereafter the cell suspensions were layered upon a sucrose cushion (9 ml) composed of 15% sucrose in PBS containing unlabeled drugs of the above concentrations, followed by centrifugation at 4500 x g for 10 min. The internalized labeled drugs were determined radiochemically in the sedimented cell pellet.

The I₅₀ values for both ligands were determined with the electrophoretically homogeneous enzyme (27) as follows. The reaction mixture, in a volume of 50 µl, contained 50 mM Tris-HCl (pH 8.0),

50 mM KCl, 10 mM 2-mercaptoethanol, 0.1 mM [32 P]-NAD (400,000 cpm) with or without 10 μ g of coenzymic DNA (27) and varying concentrations of the two drugs (between 0.01 and 1 mM) and 83 nM ADPRT. The reaction was started by the addition of ADPRT and incubation at 25 °C carried out for two minutes. The autopoly-ADP-ribosylated enzyme product was determined radiochemically as reported (27).

Virus inhibition assays: These were carried out in microplate cell cultures and consisted of either (a) comparing the evolution of cytopathic end-point titers of serial 2-fold dilutions of HIV in the presence of varying concentrations of drug with those obtained in its absence, or (b) comparing, on the fourth culture day, the cumulative production of cytopathology (syncytia) and p24 by each of a series of virus dilutions in the presence of drug with that occurring in the absence of drug. HIV titrations were performed in triplicate wells of flat-bottomed microtiter plates (Costar, Cambridge, MA) each containing 5×10^4 polybrene-treated AA-2 cells in a total volume of 200 μ l (23). End-point titers, which reflected the highest HIV dilution causing syncytia, were determined daily for 4 days and were expressed as infectious units per 0.1 ml of culture fluid. In the present studies, the effective virus dilutions employed, after addition to cells, ranged from 1:100 through 1:1600 (representing from $2.0 - 0.125 \times 10^{-2}$ infectious units per cell at day 0). Measurements of p24 were done using a commercially available immunoassay kit, following procedures prescribed by the manufacturer (Coulter, Hialeah, FL).

Cell lines: The continuous human B cell line, AA-2, was derived from the Epstein-Barr virus-transformed WIL-2 cell line (28). AA-2 cells express high levels of CD4, the HIV receptor, and they are particularly sensitive to infection with the HTLV-IIIB strain of HIV which induces the formation of large, ballooning syncytia. Because of the rapidity with which these syncytia enlarge and coalesce, they become difficult to count as infection progresses. Therefore, in these studies, HIV-induced cytopathic effects in any given culture were scored by estimating the portion of affected cells as observed by phase microscopy. MT-2 cells are an HTLV-1-carrying human T cell line (29). Compared to AA-2, these cells are about 5-fold less sensitive to HIV infection and they develop more circumscribed syncytia that are readily counted after 4 days of infection. Cell viability was assessed by trypan blue dye exclusion.

The concentration of auto-poly-ADP-ribosylated and unmodified ADPRT molecules per cell were determined by quantitative techniques as described (19) except immunodot blot was substituted for gel transblot.

Results and Discussion

Drug uptake into AA-2 and MT-2 cells as well as free ADPRT and

poly-ADP-ribosylated ADPRT content of cells in the presence and absence of externally applied 6-ABP or 5-IABP were determined in separate laboratories (Tiburon Labs) from virus titration assays (Univ. of Maryland), because of biohazard safety reasons. Intracellular drug concentrations are given in Table I, and it is evident that at equilibrium about one third of externally applied 6-ABP exists intracellularly and this value is between 8 and 9% of extracellularly applied 5-IABP. At 6 mM extracellular 6-ABP concentration (see Fig. 1) this molecule accumulated intracellularly at twice of its extracellular levels in AA-2 cells. A probable reason is that at higher concentrations of 6-ABP the rate of its oxidation by cellular enzymes to 6-nitroso-1,2-benzopyrone (33) was accelerated and the subsequent intracellular chemical reduction of the nitroso compound to the amino derivative by glutathione may have provided an acceleration of 6-ABP transport at the expense of reduced glutathione (unpublished results). Enzyme inhibitory (I_{50}) values for both drugs were determined in vitro with highly purified ADPRT (22) in the absence (31) and presence of coenzymic DNA (27) and these values are also listed in Table I. As we show here (Table II) and have also reported (19), ADPRT in intact cells exists in both ADP-ribosylated and free forms. Consequently I_{50} values without coDNA relate to cellular enzymatic activities of the free enzyme, whereas I_{50} assayed with coDNA are relevant to cellular ADPRT that is activated by DNA termini (cf. 21). During viral integration (34,35) ADPRT is most probably activated by DNA breaks, thus I_{50} values determined following the addition of coDNA to purified ADPRT correlate with cellular conditions that exist after HIV infection, which could not be directly determined because of biohazard reasons. Treatment of cells for 1 h with 90 μ M MNNG, a well known agent to produce DNA breaks (cf. 21), provided a model for the activation of ADPRT and for the testing of enzyme inhibitory drugs added to intact cells (Table II). As seen from Table I intracellular accumulation of the less potent inhibitor (6-ABP) exceeded I_{50} values and for the more active inhibitor (5-IABP) the intracellular concentrations are equal to I_{50} assayed in the

presence of coDNA. Comparison of in vitro and intracellular conditions are inevitably complicated by cellular structural factors which can modify localized intracellular drug concentrations etc., thus our model studies can provide only an approximate correlation between intracellular drug levels and ADPRT inhibitory potency in situ. The feasibility of this correlation is supported by results shown in Table II. In MT-2 cells (Expts 1-3) about 25% of ADPRT exists in the poly-ADP-ribosylated form and both drugs at 3 mM or 0.3 mM respectively depressed the steady state concentration of ADPRT-bound polymers by 50% consistent with the slow turnover poly(ADP-ribose) in undamaged cells (12). Damage of DNA by MNNG (Expt 4) significantly increased the relative concentration of poly-ADP-ribosylated ADPRT and the inhibitory effect of 6-ABP was clearly augmented (Expt 5). Some increase in the total ADPRT content induced by MNNG alone (Expt 4) and a decrease by the combined effects of 6-ABP and MNNG may indicate a change in enzyme turnover, but this mechanism was not further analysed at present. Results with AA-2 cells generally reproduced the effects seen with MT-2 cells, except there was a greater variation in the total ADPRT content (compare Expts 6-8 with 9-13) and the automodified portion of ADPRT was smaller in resting cells. Significant increase of MNNG-stimulated auto-ADP-ribosylation of ADPRT (Expts 8, 12) was markedly depressed by either 3 or 6 mM 6-ABP applied extracellularly. The effects of 0.3 mM 5-IABP are not shown in all experimental variations because they were essentially the same as that of 3 mM 6-ABP shown with MT-2 cells (Expts 3, 4).

As summarized in Table III, both 3 mM 6-ABP and 0.3 mM 5-IABP markedly depressed HIV-induced syncytia formation in MT-2 cells, indicating a profound inhibition of HIV replication. However there was no detectable change in p24 production during HIV replication and no drug effect on this presumed indicator of HIV propagation. Since mechanisms of HIV replication are as yet incompletely understood (34,35) the discrepancy between p24 production and syncytia formation cannot be resolved at present.

Both p24 and syncytia formation were greatly depressed by the drugs when HIV replication was studied in AA-2 cells (Table IV) and the sustained inhibition of HIV replication correlated with the concentration of 6-ABP, inhibition being complete at 6 mM external drug concentration (Fig. 1). It is noteworthy that the apparent intracellular inhibition of MNNG-activated ADPRT by 3 or 6 mM extracellular 6-ABP concentration was nearly the same whereas 6 mM 6-ABP completely abrogated HIV replication (Fig. 1). It is possible that results obtained with MNNG as a DNA damaging agent (Table II) are not readily comparable to those obtained with virus-infected cells. Furthermore intracellular inactivation of ADPRT by selective zinc ejection by the 6-nitroso-1,2-benzopyrone product of 6-ABP (33), that is formed from 6 mM 6-ABP, may be more effective biologically than the cellular action of reversible enzyme inhibitors. Apart from a 30-40% depression in the rate of cell growth, 6-ABP and its 5-iodo analog had no morphologically detectable toxic effects on MT-2 or AA-2 cells. Since both of these cell lines are transformed and we have reported a selective cytostatic effect of ADPRT ligands on transformed cells (3,32) the partial depression of cell growth of AA-2 and MT-2 cells was predictable. Antiviral effects of ADPRT ligands in normal human lymphoblasts will be reported elsewhere (manuscript in preparation). Our present data are consistent with a positive correlation between cellular ADPRT inhibition or inactivation and anti-HIV effects of two ADPRT ligands. As with practically all drug effects in cellular systems, alternate sites of action of both 6-ABP and 5-IABP cannot be rigorously ruled out. However all our attempts to date to demonstrate a direct inhibitory action of these drugs on enzymatic components of DNA synthesis (32) and on a variety of cellular signal pathways failed (unpublished results) and the only enzymatic component that was directly affected was ADPRT. Although the drug-induced inhibitory binding of ADPRT to RT (22) cannot be excluded among cellular mechanisms of action of 6-ABP and 5-IABP, the concomitant involvement of complex nuclear effects of ADPRT ligands (1-18) imply that several nuclear DNA sites may also participate in the

anti-HIV activity of ADPRT ligands, placing these agents in a unique category of potential anti-HIV drugs. In support of a genomic mechanism is the inhibition of UV-induced HIV gene expression by ADPRT inhibitors (36).

Acknowledgments

This research was supported by 1P01-NS-2-6665 Comprehensive AIDS Research Center grant of NIH (to G.A.C.) and by AFOSR 89-0231 (to E.K.), and by funds of the Octamer Research Foundation.

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Figure legend

Figure 1. Effect of 6-ABP on the rate of HIV replication in AA-2 cells. HIV was titrated in the presence and absence of 6-ABP. Serial 2-fold dilutions of virus were prepared in standard cell culture medium. In wells of 96-well flat-bottomed microtiter plates, 50 μ l aliquots of each dillution were admixed, in triplicate, with 50 μ l of the same medium containing either 24 mM 6-ABP or 12 mM 6-ABP or no drug (control). Immediately thereafter, 100 μ l of a suspension of AA-2 cells (5×10^5 per ml) were added to each well, producing final drug concentrations of 6 mM and 3 mM, respectively. In control cultures, and those containing 3 mM 6-ABP, HIV titers (infectious units) were determined daily for 4 days based on the appearance of cytopathology.

Fig. 1

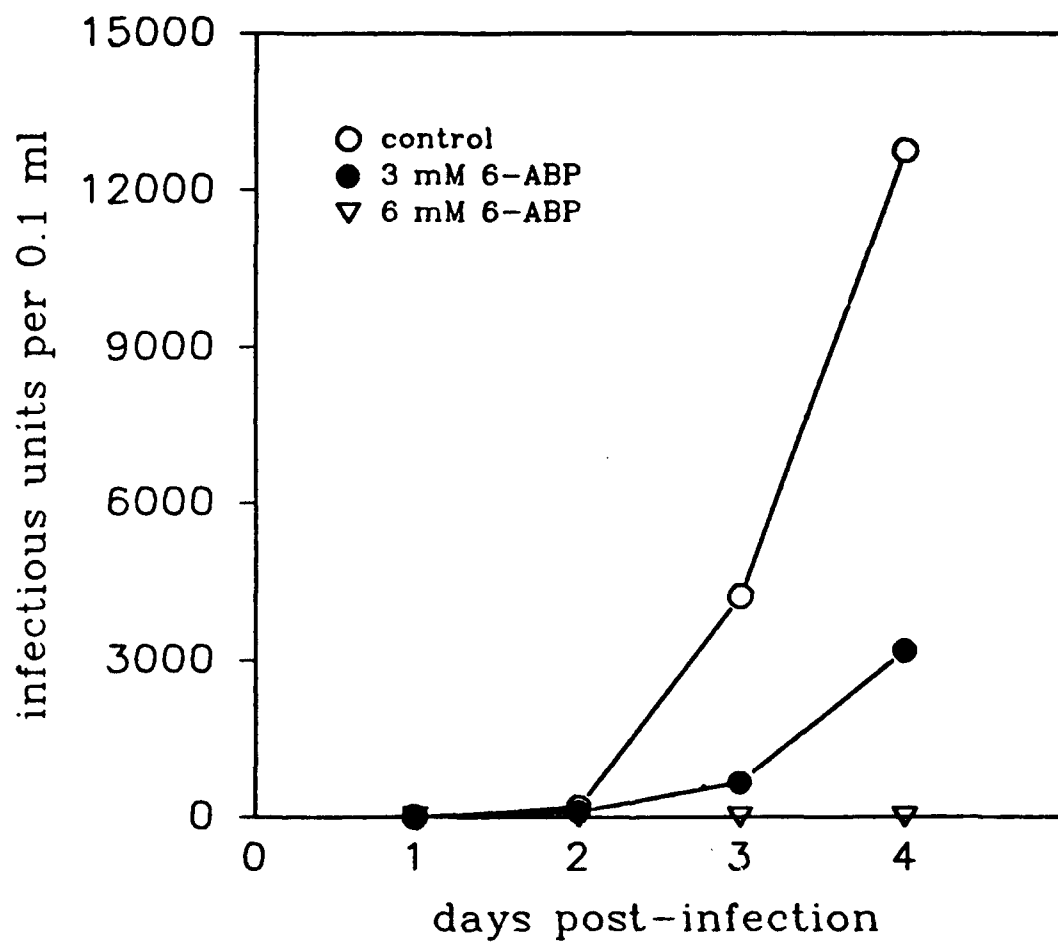


Table I

Intracellular drug concentrations at equilibrium (18 h) and I_{50} values.

Cell type	Drug	<u>Drug concentration, μM</u>		<u>I_{50}, (μM)</u>	
		external	internal	no DNA	+DNA
AA-2	6-ABP	3,000	850 ($\pm 10\%$)	30 ($\pm 15\%$)	350 ($\pm 10\%$)
"	"	6,000	14000 ($\pm 20\%$)		
"	5-IABP	300	35 ($\pm 20\%$)	2 ($\pm 15\%$)	40 ($\pm 10\%$)
MT-2	6-ABP	3,000	640 ($\pm 25\%$)		
"	5-IABP	300	26 ($\pm 20\%$)		

Table I. Intracellular drug concentration and I_{50} values were determined in triplicate, as given in Materials and Methods. Variations from the mean value are expressed as $\pm\%$.

Table II

Intracellular concentration of unmodified and poly-ADP-ribosylated ADPRT in intact cells and the effect of drugs.

Exp. No.	Cell type	Experimental conditions	Unmodified ADPRT ng/10 ⁵ cells	Poly-ADP-ribosylated ADPRT, ng/10 ⁵ cells
1	MT-2	control	2.5(±15%)	0.65(±15%)
2	"	3 mM 6-ABP	2.1(±15%)	0.31(±15%)
3	"	0.3 mM 5-IABP	2.1(±15%)	0.26(±15%)
4	"	1 h exposure to 90 µM MNNG	1.8(±15%)	2.1(±15%)
5	"	3 mM 6-ABP + 90 µM MNNG	1.4(±20%)	0.6(±15%)
6	AA-2	control	6.3(±10%)	0.25(±15%)
7	"	3 mM 6-ABP	7.1(±15%)	0.20(±15%)
8	"	90 µM MNNG	3.7(±10%)	2.0(±10%)
9	"	3 mM 6-ABP + 90 µM MNNG	3.4(±10%)	0.45(±20%)
10	"	control	4.8(±15%)	0.24(±15%)
11	"	6 mM 6-ABP	4.0(±20%)	0.16(±20%)
12	"	90 µM MNNG	3.0(±20%)	2.10(±10%)
13	"	6 mM 6-ABP + 90 µM MNNG	2.4(±18%)	0.22(±20%)

Table II. Intracellular concentrations of ADPRT and its poly-ADP-ribosylated form were determined immunologically in "citric acid" nuclei as described (19). The phenyl boronate column-retained ADPRT is a direct assay for the quantity of poly-ADP-ribosylated enzyme. Drug treatment consisted of incubation of cells under exactly same conditions as described in Tables III and IV. When DNA-damage was elicited, this was done by incubation with 90 µM MNNG for 1 h. Citric acid nuclei were prepared from 20-30 x 10⁶ control- and drug-treated cells. Assays were done in triplicates and deviation from the mean value is given as ±%.

Table III

Effect of 6-ABP (3mM) and 5-IABP (0.3 mM) on HIV replication in MT-2 cells.

Drug treatment ¹	Input infectious HIV units x 10 ⁻² per cell (day 0)	pg/ml p24 output (day 4) ²	Syncytia production counts ³ (%inhib.)	
none	2.0	2,187	150	
"	1.0	1,565	83	
"	0.5	840	26	
"	0.25	349	6	
"	0.125	170	4	
6-ABP	2.0	2,137	2	(99)
"	1.0	1,428	0	(100)
"	0.5	599	0	(100)
"	0.25	350	0	(100)
"	0.125	191	0	(100)
5-IABP	2.0	2,226	36	(76)
"	1.0	1,747	15	(82)
"	0.5	773	0	(100)
"	0.25	460	0	(100)
"	0.125	205	0	(100)

¹ Drug was present in the medium throughout the 4-day culture period.

² Mean values from determinations on duplicate samples. Numerical differences between duplicates did not exceed 5%.

³ Mean number of syncytia in duplicate infected microtiter plate cultures (5 x 10⁴ MT-2 cells per well) on day 4.

Table IV

Effect of 6-ABP (3 mM) and 5-IABP (0.3 mM) on HIV replication in AA-2 cells.

Drug treatment ¹	Input infectious HIV units x 10 ⁻² per cell (day 0)	p24 output (day 4) ²		Syncytia score ³
		pg/ml	(inhib.)	
none	2.0	6,340		3
"	1.0	5,083		2
"	0.5	3,065		2
"	0.25	1,444		1
"	0.125	758		1
6-ABP	2.0	2,183	(65)	0
"	1.0	1,451	(71)	0
"	0.5	757	(75)	0
"	0.25	375	(74)	0
"	0.125	199	(74)	0
5-IABP	2.0	6,105	(4)	2
"	1.0	2,810	(45)	1
"	0.5	748	(76)	1
"	0.25	602	(58)	<1
"	0.125	200	(74)	<1

¹ Drug was present in the medium throughout the 4-day culture period.

² Mean values from determinations on duplicate samples. Numerical differences between duplicates did not exceed 5%.

³ Based on extent of cytopathology (syncytia) present in duplicate infected microtiter plate cultures (5x10⁴ AA-2 cells per well) on day 4 and scored as follows: 0=no syncytia; 1=1-25% syncytia; 2=26-50% syncytia; 3=51-75% syncytia.